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Designed and Developed Delivery Systems Containing Extracted Astaxanthin from Crawfish, *Procambarus clarkii*, Using a Novel Combined Ethanol Flaxseed Oil Ultrasound Assisted Closed Extraction System and Its Anticancer Activity in Vitro

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**DESIGNED AND DEVELOPED DELIVERY SYSTEMS
CONTAINING EXTRACTED ASTAXANTHIN FROM CRAWFISH,
PROCAMBARUS CLARKII, USING A NOVEL COMBINED
ETHANOL FLAXSEED OIL ULTRASOUND ASSISTED CLOSED
EXTRACTION SYSTEM AND ITS ANTICANCER ACTIVITY *IN
VITRO***

A Dissertation

Submitted to the Graduate Faculty of the
Louisiana State University and
Agricultural and Mechanical College
in partial fulfillment of the
requirements for the degree of Doctor of
Philosophy

in

The School of Nutrition and Food Sciences

by

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August 2020

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First and foremost, I want to give thanks to God for his grace and mercy in allowing me to accomplish my endeavors.

This study is wholeheartedly dedicated to my beloved mother, who never gave up on me throughout the tumultuous challenge and gave me strength to keep going when I was knocked down, who continued to provide spiritual, emotional, and moral support.

To my siblings, relatives, friends, classmates, mentors, and major advisor who shared their words of advice and encouragement to finish my study.

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LIST OF ABBREVIATIONS AND ACRONYMS

#UAE	# Grams Crawfish Processing Byproducts Used with # Grams Flaxseed Oil for Ultrasound Assisted Extraction
AC	Astaxanthin Content
AGEs	Advanced Glycation End Products
ALA	Alpha-Linolenic Acid
AX	Astaxanthin
AXC	Astaxanthin Concentration
AXE	Astaxanthin Lecithin Flaxseed Oil Emulsions
CB	Crawfish Processing Byproducts
CFO	Flaxseed Oil Only with Ultrasound Assisted Extraction Using 25 Grams of Crawfish Processing Byproducts
DS	Delivery System
DMs	Diabetes mellitus
DM	Degree of methylation
DMEM	Dulbecco's modified Eagle medium
DHA	Docosahexaenoic
DPPH	2,2-diphenyl-1-picrylhydrazyl radical
E	Ethanol
EMT	Epithelial-to-mesenchymal transition
EPA	Eicosapentaenoic Acid
ER	Estrogen Receptor
FA	Fatty Acid
FBS	Fetal Bovine Serum

FFA	Free Fatty Acid
FO	Flaxseed Oil
FOAX	Flaxseed Oil Containing Astaxanthin
FOCAX	Flaxseed Oil Containing Crawfish Astaxanthin From Conventional Extraction
FOCAXUAE	Flaxseed Oil Containing Crawfish Astaxanthin From Ethanol and Ultrasound Assisted Extraction
FOSAX	Flaxseed Oil Containing Shrimp Astaxanthin From Conventional Extraction
G	Gelatin
GOM	Gulf of Mexico
GPx	Glutathione Peroxidase
GSH	Glutathione
H_2O_2	Hydrogen Peroxide
HFO	Heated Flaxseed Oil
HER2	Human Epidermal Growth Factor
HDL	High Density Lipoproteins
HMP	High-Methoxyl Pectin
*OH	Hydroxyl Radical
HPMC	Hydroxypropyl Methylcellulose
L*	Fatty Acid Radical, Alkyl Radical
LC	Lecithin: Flaxseed Oil Control
LDL	Low Density Lipoproteins
LMP	Low-Methoxyl Pectin
MET	Mesenchymal-to-epithelial transition

MDA-MB-231	M.D. Anderson Metastasis Breast cancer 231
NFO	Ethanol Only During Ultrasound Assisted Extraction Using 25 Grams of Crawfish Processing Byproducts (
P	Pectin
PR	Progesterone Receptor
PV	Peroxide Value
O ₂ ⁻	Superoxide Anion Radical
RCV	Relative Cell Viability
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SB	Shrimp Processing Byproducts
SGF	Simulated Gastric Fluid
SIF	Simulated Intestinal Fluid
SL	Sunflower Lecithin
SOD	Superoxide Dismutase
TBA	Thiobarbituric Acid
TBARs	Thiobarbituric Acid Reactive Substances
TFC	Total FOCAxUAE Content in PGDS
TG	Triglycerides
TNBC	Triple-Negative Breast Cancer
UAE	Ultrasound Assisted Extraction
UTCB	Flaxseed Oil Only that was Pumped through the System with 25 Grams of Byproducts to Determine the Effects on the Oil from Pumping Only the Oil through the System

UTO	Flaxseed Oil Only that was Pumped through the system by itself (no byproducts) to determine the effects on the oil from pumping only the oil through the system
VCEAC	Vitamin C Equivalent Antioxidant Capacity

ABSTRACT

Crustacean processing in Louisiana generates vast amounts of byproducts, including crawfish processing byproducts (CB), whose disposal can be problematic unless utilized alternatively. The objective of this investigation was to design and develop a delivery system (DS) containing extracted astaxanthin (AX) from CB using a novel combined ethanol flaxseed oil (FO) ultrasound assisted extraction (UAE) method and evaluate its quality characteristics. Additionally, anticancer effects of AX were evaluated. Investigations consisted of three studies.

Proximate composition, oxidative stability, and antioxidant capacity of AX from CB extracted both conventionally and by UAE was determined. AX concentration conventionally extracted from CB (FOCAX) was 0.0919 mg g^{-1} of FO. The combined UAE ethanol FO extracted AX from CB (FOCAXUAE) was 1.9 mg g^{-1} of FO with antioxidant capacity (71.81 ± 0.42) significantly greater than FOCAX (63.16 ± 0.22). FOCAXUAE was selected for use in further studies.

A pectin gelatin DSs (PGDS) for controlled release of extracted AX was developed. Releasing profiles of several formulations containing high degrees of methylation (HMP) or low degrees of methylation (LMP) pectins were evaluated. The *in vitro* release study investigated release of AX in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF). However, desired release characteristics were obtained with the addition of hydroxypropyl methylcellulose (HPMC). Swelling index of both HMP and LMP formulations with HPMC in SIF after 180 minutes were 126.46% and 90.61%, respectively. Other formulations failed to swell in SIF, with exposure to low pH SGF resulting in all formulations rapidly releasing FOCAXUAE.

Anticancer properties were considered. A control (sunflower lecithin and FO in DMEM, Dulbecco's modified Eagles medium) in the ratio (0.0025:0.005:1) on the viability of MDA-MB-

231 cell lines showed RCV (relative cell viability) of 79.37 ± 0.87 % (RCV of 97.37 ± 0.06 %, normalized to DMEM alone). RCV of MDA-MB-231 cells treated with AX ($3 \mu\text{M}$, $5 \mu\text{M}$, and $7 \mu\text{M}$) had significantly decreased (46.31 ± 0.81 %, 42.2 ± 0.65 %, and 55.51 ± 0.19 %, respectively).

Cell densities decreased in some groups treated with AX, showing fewer cells with mesenchymal-like shapes, suggesting reversal of the epithelial-to-mesenchymal transition (EMT). This research indicates high amounts of AX extracted from CB by UAE has cytotoxic activity that could be incorporated into polymer-based DS.

CHAPTER 1 LITERATURE REVIEW

1.1 Louisiana seafood

The State of Louisiana is known as the “Sportsman’s Paradise” due to its beautiful landscape for hunting and waters for fishing, that attract tourist from around the world. In addition to the thrill of hunting and fishing, there are a plethora of restaurants throughout Louisiana that serve up that delicious food that many people long to experience. From an economic standpoint, Louisiana seafood industry generates almost \$2 billion in sales annually, while supporting over 33,000 jobs, not including recreational fishing (Gaudé, 2019).

1.1.1 Red swamp crawfish (*Procambarus clarkii*)

In 1983 the red swamp crawfish (*Procambarus clarkii*) Louisiana crawfish, was designated as the state's crustacean (Schedler, 2011). In North America, Louisiana dominates the crawfish industry, accounting for about 90%-95% of the total production in the United States. Crawfish are an important commercial species cultured with high market values exceeding \$170 million annually (Osti, Bampasidou, & Fannin, 2018). In the southern region of the United States crawfish are regarded more communally and have had a greater impact economically than any other place where they exist in the world. In addition to the edible portions of crawfish, several other components can be derived from crawfish byproducts like flavor compounds, chitin, chitosan and carotenoids like astaxanthin (Meyers & Bligh, 1981; Hong K No & Meyers, 1989; Hong K No, Meyers, & Lee, 1989; Tanchotikul & Hsieh, 1989).

Even with the high market value associated with crawfish they are much appreciated in many states across the United States. The market value of Louisiana crawfish exceeds more than \$170 million annually, reportedly adding \$300 million to the state’s economy (Johnson, 2019). Crawfish are frequently sold boiled ready to eat, live in sacks for crawfish boils, or as frozen

peeled tail-meat. In times of oversupply, large crawfish remain in the live market and sometimes smaller crawfish are produced for meat (Editor, 2010). These undersized, also termed underutilized crawfish, have very little market value and are often discarded. Subsequently, this has resulted in an increase in the production of crawfish waste, or byproducts from processing. The utilization of undersized crawfish or the shells from peeling operations for the extraction of astaxanthin may increase the economic value of crawfish.

The Louisiana crawfish culture industry has been reported to be the largest crustacean farming operation in the United States for more than 30 years. Much, if not most, of the crawfish production is consumed and or processed in the state within two relatively concentrated areas, Breaux Bridge, LA and Crowley, LA (Laudun, 2016; Mulcahy, Naquin, & Harper, 2019; Poudel, 2016; Richardson, 2015). Therefore, there should be a stable supply of byproducts for conversion into alternative products. There is a great potential in the crawfish processing industry to convert previously discarded byproducts into valuable products. Please note that the term byproducts is not clearly defined to distinguish it from waste and it is often identified as leftovers that are not ordinarily saleable, rather than potentially valuable components that can be used with proper treatment.

1.2 Antioxidants

The prevention of diseases is important to health and well-being. People are encouraged to eat a well-balanced diet and to exercise, in efforts to avoid illness. Antioxidants are reported to play a noteworthy role in both the prevention and treatment of diseases. Antioxidants are naturally found in fruits and vegetables or can be additives used to preserve food from “farm to table”, that prevent deterioration in storage and processing (Griffiths et al., 2016). Antioxidants

may also be present in health and beauty products like face creams and shampoo. They function to combat oxidation of compounds in products that result in the loss of functional quality.

All biological systems exist in redox equilibrium, balancing reducing and oxidative reactions to meet necessary conditions for life (Gaschler & Stockwell, 2017). An imbalance of the aforementioned redox equilibrium results in potentially severe biological damage. In the case of aerobic organisms, oxidation is a normal metabolic process in which energy is produced through a series of reactions that involve oxidants. When there is an overproduction of ROS, such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical (HO^*) or reactive nitrogen species (RNS), the biological redox balance called redox equilibrium is tilted, creating oxidative stress (Figure 1.1). Antioxidants function to combat oxidative stress in the body. These include non-enzymatic scavengers such as vitamin C and E, glutathione (GSH), lipoic acid, carotenoids and iron chelators, as well as enzymatic scavengers such as superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and peroxiredoxins (Redza-Dutordoir & Averill-Bates, 2016). Conversely, an overproduction of reductants leads to reductive stress (Woods, Wilund, Martin, & Kistler, 2012).

Biological Redox Balance

A delicate balance between oxidizing compounds and reductants

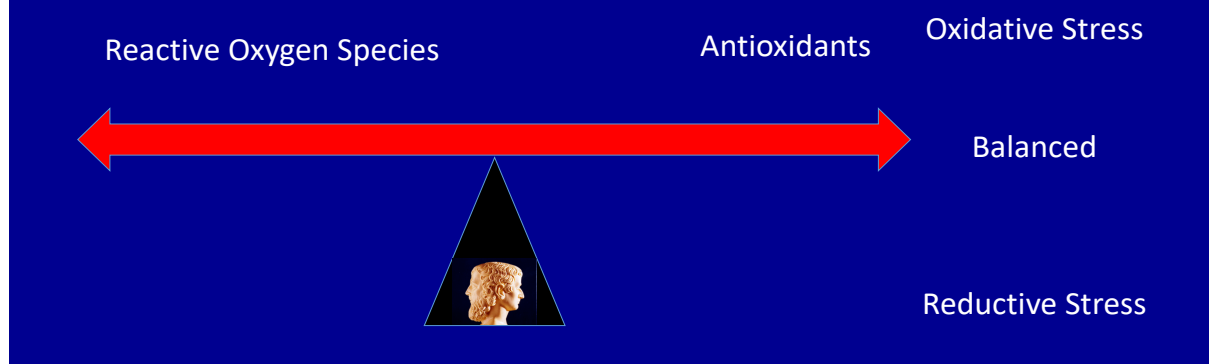


Figure 1.1. Balance of redox equilibrium. Source: Spickett, Fedorova, Hoffmann, and Forman (2015)

The beneficial effects of natural antioxidants found in fruits and vegetables are related to three major groups: carotenoids, phenolics, and vitamins. Of the three categories, carotenoids are known as lipophilic antioxidants, with the others being hydrophilic (Thaipong, Boonprakob, Crosby, Cisneros-Zevallos, & Hawkins Byrne, 2006). Antioxidants compounds may be produced in the body or obtained from food or supplements, and may be either natural or man-made. As mentioned earlier, antioxidants prevent foods from spoiling, Examples of natural antioxidants include SOD and GPx, both produced by the body, and chemical antioxidants, such as the carotenoids, vitamin E, lutein, and astaxanthin, all dietary. Antioxidants can prevent certain types of cell damage or lipid oxidation induced by oxidative stress. They have also been used as reactive oxygen species scavengers to eliminate oxidative stress.

Several carotenoids act as potent antioxidants. Antioxidants have various health benefits, including prevention of ocular disease, reduction of inflammation, prevention of Alzheimer's disease, reduction of the risk of stroke, and the improvement of blood glucose in diabetic therapy

(Bahonar, Saadatnia, Khorvash, Maracy, & Khosravi, 2017; Behl & Moosmann, 2002; C. Li et al., 2017; N. Singh & Ghosh, 2019). Recently antioxidants were reported to reduce the incidence of coronary heart disease and prevent cancers. A more detailed discussion follows.

1.2.1 Reduction of inflammation

Oxidative stress triggers the release of cell signaling proteins, chemokines, cytokines, and activation of their receptors as well as lipid peroxidation in the human body. Resulting in the generation of pro-inflammatory, which are not an indication of the abnormalities directly caused by the disease but are due to the activation of the body's defense mechanisms. However, this self-defense mechanism, collectively termed inflammation, may become harmful if it transforms into chronic inflammation (Levine, Mizushima, & Virgin, 2011). For example, *Helicobacter pylori*, a Gram-negative bacterium, causes chronic gastritis. The pathogenesis is partly caused by an immunological response and affects roughly half of the world population. Astaxanthin was shown to attenuate gastritis caused by *H. pylori* infections by inhibiting the growth of the bacterium, to ultimately suppress gastric inflammation (Bennedsen, Wang, Willén, Wadström, & Andersen, 2000). Additionally, Kang and Kim (2017) found that the inflammatory response impairment stimulated by *H. pylori*-infected gastric tissues is abated by astaxanthin modulating the cellular response to shift the activation of T helper cell type 1 response towards T helper type 2 response.

1.2.2 Cancer prevention

Not only does the intake of dietary fats (i.e. saturated fats) promote the development of coronary heart disease, they have also been linked to the development of certain cancers because of the chemical changes they can undergo during thermal processing (Kolonel, 2001; Norat et al., 2005). An approach to combat such deleterious effects has created much interest foods for

cancer prevention, an interest in bioactive compounds, like antioxidants, from fruits and vegetables has been promising. The importance of oxidative damage in the etiology of human cancer and the need for intrinsic and dietary antioxidants was first studied by the American biochemist, Dr. Bruce Ames. Most notably, Dr. Ames is known for the development of the Ames test, which is an assay for mutagens and their potential to be carcinogenic. Researcher Totter (1980) reported that oxygen radicals and lipid oxidation agents are contributors to DNA damage that results in diseases like cancer. He also reported that tocopherol, a radical trap in lipid membranes, was used in a variety of oxygen-related diseases and that it was capable of ameliorating the damage and carcinogenicity of several quinones. In the same report, it was mentioned that β -carotene in green and yellow vegetables in the diet may be anticarcinogens and inhibits tumorigenesis in several cancer models *in vivo* (Shree et al., 2017; Totter, 1980). The main carotenoid found in aquatic animals, microorganisms and seafood, astaxanthin has attracted considerable attention in part due to it being more potent than that of β -carotene and other carotenoids as it relates to its antitumoral activity (Palozza et al., 2009)

Several epidemiological studies indicate an association between nutritional habits and prevention of several types of cancer (Campbell, Newton, Jacobs, Pollak, & Gapstur, 2019; Emenaker & Vargas, 2018; Susan T Mayne, Playdon, & Rock, 2016). The relationship between nutrition and cancer dates back before the 1940s, when researchers first considered that restricting diet, namely a caloric restriction, resulted in delayed growth and development of mouse carcinomas (Bischoff & Long, 1938; Sugiura & Benedict, 1936; Tannenbaum, 1940). Nowadays, with the advancements in the fields of molecular biology, medicine, nutrition and technology since the mid 20th century, researchers have turned the focus from not only caloric restriction but to the restriction of foods thought to promote cancer and foods that contain cancer

preventing components (antioxidant micronutrients).

1.3 Xanthophyll carotenoid: astaxanthin

Carotenoids, are non-polar, fat-soluble tetraterpenes consisting of linked isoprene units found in nature and responsible for imparting color to pigments of many fish and shellfish (Lindshield, 2012; Martínez-Delgado, Khandual, & Villanueva-Rodríguez, 2017; N. M. Sachindra, Bhaskar, & Mahendrakar, 2005). Carotenoids are classified in two ways, whether or not it is capable of being converted to Vitamin A, and on structural differences and polarity. The oxygenated carotenoids are known as xanthophylls, whereas hydrocarbon carotenoids are known as carotenes (Lindshield, 2012).

Natural pigments are found widespread in all living matter including mammals, fungi, invertebrates, and marine algae (Delgado-Vargas, Jiménez, & Paredes-López, 2000). The carotenoid family consists of more than 700 pigments synthesized *de novo* in higher plants, mosses, algae, bacteria, and fungi whose structures are derived from the acyclic isoprenoid lycopene (I. Higuera-Ciapara, Felix-Valenzuela, & Goycoolea, 2006; Jackson, Braun, & Ernst, 2008; Khachik, Beecher, & Smith Jr, 1995). There are two important classifications of carotenoids; carotenes, a subclass composed of only hydrocarbons, and xanthophylls, a subclass consisting of oxygenated derivatives of carotenes (Jackson et al., 2008).

Several carotenoids act as potent antioxidants in humans and have been investigated because of their ability to lessen the damaging effects of ROS. A carotenoids ability to efficiently scavenge free radicals and to absorb light in the visible region is due to the multiple conjugated double bonds contained within its structure (Lindshield, 2012). Plants employ carotenoids light harvesting ability in the complex that captures light for photosynthesis. Carotenoids are also found in bacteria, yeasts, molds crustaceans and bird feathers. Mammals do not synthesize

carotenoids; therefore, they must be obtained through diet.

Additionally, seafood can also be a source of marine natural pigments (Ferraro et al., 2010). Chlorophylls, carotenoids and phycobiliproteins are three basic classes of natural pigments found in seafood (Noomhorm, Ahmad, & Anal, 2014). Crawfish and shrimp byproducts, contain high levels of health benefitting bioactive compounds, such as proteins, carotenoids, minerals, pigments and flavor compounds (Ibrahim, Salama, & El-Banna, 1999). Astaxanthin, a xanthophyll carotenoid synthesized by plants and algae and found in crustaceans, is a well-known antioxidant with multiple health promoting qualities has gained much attention recently (Ambati, Phang, Ravi, & Aswathanarayana, 2014).

Due to its outstanding antioxidant activity, astaxanthin is capable of providing protection against cardiovascular diseases, different types of cancer, some diseases of the immunological system, macular degeneration and age-related cataracts, the latter two being related to light-induced oxidative processes in the eye (Guerin, Huntley, & Olaizola, 2003; I. Higuera-Ciapara et al., 2006). Some research has shown the ability of astaxanthin to ameliorate triglycerides (TG) and high-density lipoproteins (HDL) in animal studies, thus potentially improving lipid profiles in humans (H. Yoshida et al., 2010). Additionally, the structure of astaxanthin makes it a more potent anti-oxidant than β -carotene (Jackson et al., 2008).

Albeit astaxanthin having the capability of exerting antioxidant effects, it does so without being a pro-oxidant. Pro-oxidants induce oxidative stress by hindering antioxidant systems or generating reactive oxygen species. It is known that reactive oxygen species are the underlying cause of many diseases and illnesses.

1.3.1 Chemical Structure

The carotenoid family consists of more than 700 pigments synthesized *de novo* in higher

plants, mosses, algae, bacteria, and fungi divided into two groups, whose structures are derived from the acyclic isoprenoid lycopene, seen in Figure 1.2 (I. Higuera-Ciapara et al., 2006; Jackson et al., 2008). The two groups are based on the chemical elements they contain in their molecules: carotenes, which are composed of only hydrocarbons, and xanthophylls, consisting of oxygenated derivatives of carotenes (Jackson et al., 2008). The oxygen present in xanthophylls may be present as hydroxyl (OH) groups, carbonyl (C=O) groups or as a combination of both. Astaxanthin, a xanthophyll carotenoid, which is synthesized by plants and algae from lycopene by oxidative functionalization, has strong antioxidant properties (Ambati et al., 2014). The presence of OH and C=O in each ionone ring contribute to the strong antioxidant capacity of astaxanthin and its ability to be esterified.

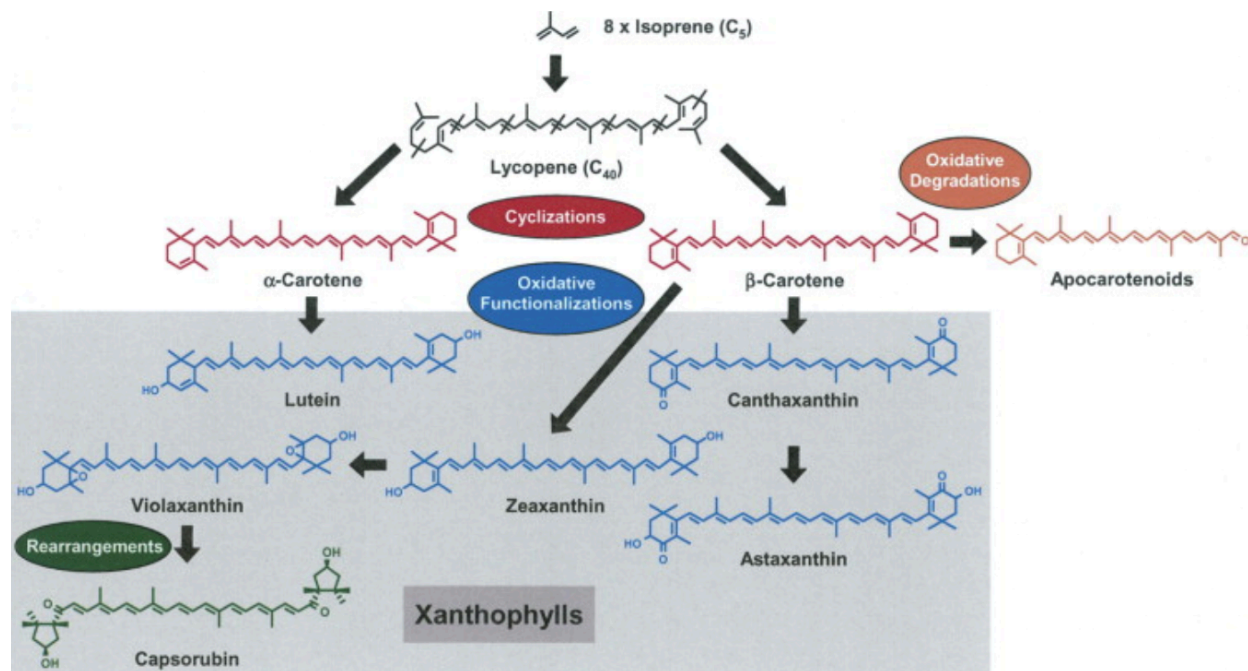


Figure 1.2. Carotenoid biosynthesis from lycopene. Source: Jackson et al. (2008).

Astaxanthin (3,3'-dihydroxy-β, β-carotene-4,4'-dione) is carotenoid pigment containing 11 conjugated double bonds and two hydroxyl groups, Figure 1.3 (Martínez-Delgado et al., 2017). The long chain of conjugated double bonds (polyene) and two terminal ring systems

(ionone rings) determine its chemical characteristics and light absorption properties of astaxanthin. In nature, astaxanthin is found in three trans isomer, however the 3*S*, 3'*S*', seen in Figure 1.4, is the most common (Coral-Hinostroza, Ytrestøyl, Ruyter, & Bjerkeng, 2004; I. Higuera-Ciapara et al., 2006). Due to the different chirality at C-3, 3', astaxanthin has three R/S (optical) isomers including two enantiomers (3*S*,3'*S*) and (3*R*,3'*R*) and the meso form (3*R*,3'*S*) (Figure 1.4).

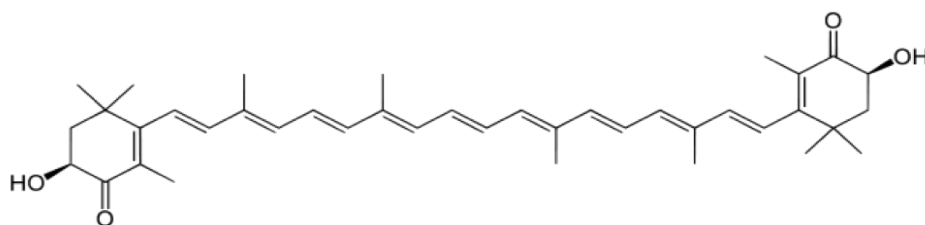


Figure 1.3. Chemical structure of astaxanthin. Source: Lorenz and Cysewski (2000)

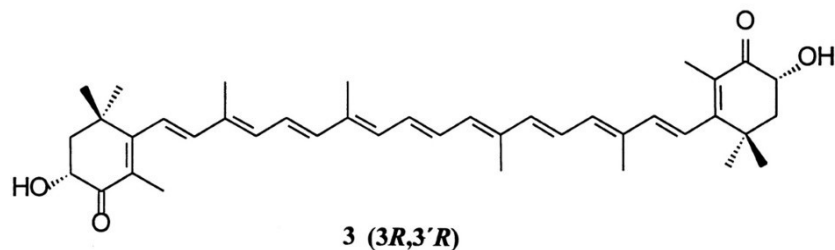
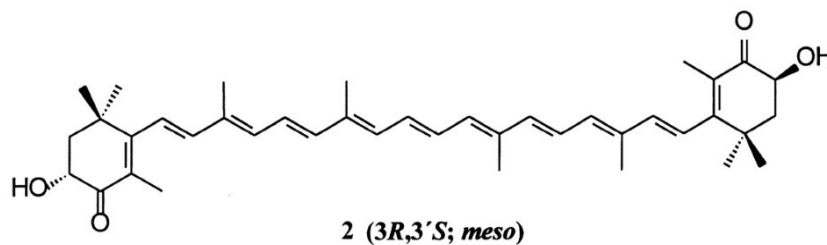
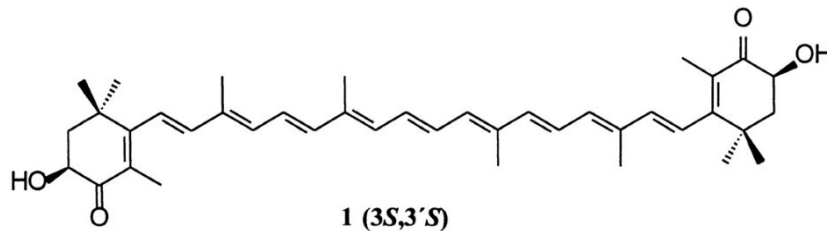


Figure 1.4. Structures of optical isomers all-E-astaxanthin. Source: Coral-Hinostroza et al. (2004)

All-E configuration (a, straight chained) and the three Z configurations (b, c, and e, bent chains) in Figure 1.5 below, have greater solubility and improved oral bioavailability (Østerlie, Bjerkeng, & Liaaen-Jensen, 2000).

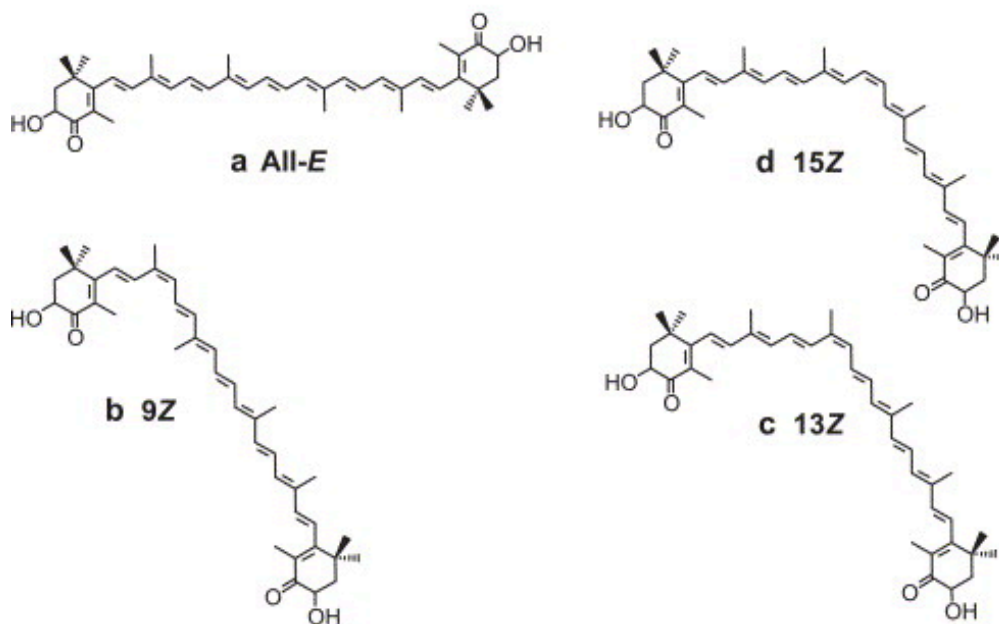


Figure 1.5. Geometrical isomers all-E-, 9Z-, 13Z- and 15Z-astaxanthin. Source: Østerlie, Bjerkeng, and Liaaen-Jensen (1999)

1.3.2 Sources of Astaxanthin

1.3.2.1 Microorganisms

The primary source of the carotenoid astaxanthin present in aquatic animals is algae, namely *Haematococcus pluvialis*, the most abundant source of natural astaxanthin (Guerin et al., 2003; Maoka, 2011; Matsuno, 2001). Astaxanthin, a red fat-soluble xanthophyll carotenoid, can be found in various microorganism (Table 1.1) including yeast, algae, bacteria (Asker, Beppu, & Ueda, 2007; J. H. Lee, Kim, Choi, Lee, & Kim, 2004; Lemoine & Schoefs, 2010; Libkind, Moliné, de García, Fontenla, & van Broock, 2008). Commercial astaxanthin is mainly from *Phaffia* yeast, *Haematococcus* and through chemical synthesis.

Table 1.1 Sources of Astaxanthin

Sources	Astaxanthin (%) on a Dry Weight Basis	References
Alphaproteobacteria		
<i>Agrobacterium aurantiacum</i>	0.01	(Yokoyama and others 1995)
<i>Paracoccus carotinifaciens</i> (NITE SD 00017)	2.2	(Borie 2007)
Chlorophyceae		
<i>Haematococcus pluvialis</i>	3.8	(Ranga and others 2009; Ranga Rao and others 2010)
<i>Haematococcus pluvialis</i> (K-0084)	3.8	(Aflalo and others 2007)
<i>Haematococcus pluvialis</i> (Local isolation)	3.6	(Torzillo and others 2003)
<i>Haematococcus pluvialis</i> (AQSE002)	3.4	(Olaizola 2000)
<i>Haematococcus pluvialis</i> (K-0084)	2.7	(Wang and others 2013)
<i>Chlorococcum</i>	0.2	(Zhang and Lee 1997; Zhang and others 1997)
<i>Chlorella zofingiensis</i>	0.001	(Wang and Peng 2008)
<i>Neochloris wimmeri</i>	0.6	(Orosa and others 2000)
Floriideophyceae		
<i>Catenella repens</i>	0.02	(Banerjee and others 2009)
Labyrinthulomycetes		
<i>Thraustochytrium</i> sp. CHN-3 (FERM P-18556)	0.2	(Yamaoaka 2008)
Malacostraca		
<i>Pandalus borealis</i>	0.12	(EFSA 2005)
<i>Pandalus clarkia</i>	0.015	(Meyers and Bligh 1981)
Tremellomycetes		
<i>Phaffia rhodozyma</i>	0.00001	
<i>Xanthophyllomyces dendrorhous</i> (JH)	0.5	(Kim and others 2005)

(table cont'd)

Sources	Astaxanthin (%) on a Dry Weight Basis	References
<i>Xanthophyllomyces dendrorhous</i> (VKPM Y2476) Ulvophyceae	0.5	(de la Fuente and others 2010)
<i>Enteromorpha intestinalis</i>	0.02	(Banerjee and others 2009)
<i>Ulva lactuca</i>	0.01	(Banerjee and others 2009)

1.3.2.2 Seafood byproducts

Astaxanthin is the primary naturally occurring pigment in marine crustaceans and in the flesh of salmonids (Felix-Valenzuela, 2001). Unable to synthesize the pigment, these marine creatures obtain the astaxanthin from their environment, or if farmed commercially, through their feed. Byproducts from crustaceans are produced during processing operations for recovery of the edible portion of crabs, crawfish, shrimp, and lobster; and consist of mineral salts, proteins, chitin, lipids, and pigments. Natural marine fishery sources of astaxanthin are salmon, trout, krill, crawfish and shrimp (Ambati et al., 2014). Astaxanthin, the predominant carotenoid found in crustacean waste is free or esterified with fatty acids (De Holanda & Netto, 2006; Kim, Perera, Rajapakse, & Kim, 2014). Crab, salmon and shrimp can serve as dietary sources of astaxanthin (Authority, 2005)

1.3.2.3 Chemical synthesis

There are 5 C40 carotenoids manufactured synthetically, from petroleum feedstocks, on an industrial scale (i.e. including β,β -carotene, lycopene, canthaxanthin, zeaxanthin, and astaxanthin) for use as nutritional supplements and for animal feed additives in poultry farming and aquaculture that are essential for the animals' growth, health and reproduction (Jackson et al., 2008). The most widely used method of producing synthetic astaxanthin is the Wittig reaction is

produced in an isomeric form similar to the form predominately found in nature (Ernst, 2002). Synthetic astaxanthin is a racemic mixture (1:2:2:1) of all three-isomers (3S, 3S'), (3R, 3R'), (3S, 3R') and (3R, 3S'), respectively) as seen in Fig 1.6 (I. Higuera-Ciapara et al., 2006). Synthetic astaxanthin is identical molecularly to that produced in living organisms, and is the main carotenoid used in the aquaculture industry throughout the world (I. Higuera-Ciapara et al., 2006).

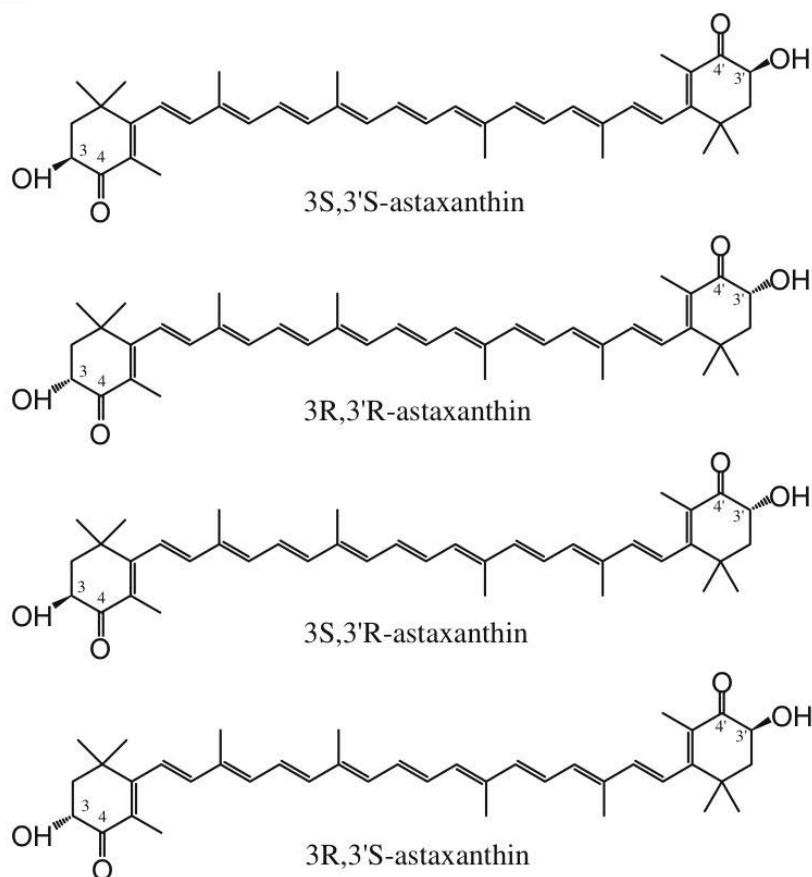


Figure 1.6. Synthetic astaxanthin stereoisomers. Source: I. Higuera-Ciapara et al. (2006)

Studies have shown that both natural and synthetic astaxanthin produce satisfactory effects, but human trials for natural astaxanthin are lacking due to production availability as evidence by several studies. Furthermore, there has not been any established recommended dose for astaxanthin daily intake, however most research findings report benefits from a daily intake

of 4mg. Despite the low cost for manufacturing synthetic carotenoids, there is a deleterious impact on the environment, and growing health concerns, there is an increasing demand for more natural sourced compounds; this has led many researchers to investigate natural sources of astaxanthin, such as crustacean byproducts (Cheng & Yang, 2016; Dalei & Sahoo, 2015; Gateau, Solymosi, Marchand, & Schoefs, 2017; Gupta, Jha, Pal, & Venkateshwarlu, 2007; Ye, Jiang, & Wu, 2008).

1.3.3 Uses of Astaxanthin

Astaxanthin has several patented applications available in feed, food, nutraceutical and pharmaceutical applications (Ambati et al., 2014). Nutraceuticals are physiologically beneficial foods that protect against chronic diseases (Hughes, Marangoni, Wright, Rogers, & Rush, 2009). AX products are available in many different forms, from capsules, soft gels, tablets and energy drinks to powder, oil, biomass, cream, oil and extracts.

Mammals, fish and crustaceans are unable to synthesize astaxanthin *de novo*, thus it must be obtained from their diet, or partly modified through metabolic reactions (Liaaen-Jensen, 1990, 1998; Maoka, 2011; Matsuno, 2001). In nature astaxanthin, biosynthesized in plants, algae, and some bacteria and fungi, is absorbed (dietary) and metabolized by mammals, fish and crustaceans (H.-M. Chen & S. P. Meyers, 1982) accumulating in the skin, muscle or exoskeleton. That results in the colors seen in flamingos, salmon, shrimp crawfish and rainbow trout (Shahidi & Synowiecki, 1991).

1.3.4 Recovery of Astaxanthin

Several techniques have been used to extract astaxanthin from microorganisms (microalgae, bacteria, and yeast), marine organisms (shrimp, crabs, crawfish and lobster) and wastewater. Such techniques include acid, alkaline, and enzymatic treatments, vegetable oils and

solvents with supercritical fluid extraction, ultrasound-assisted extraction, and ultrafiltration (Amado, González, Murado, & Vázquez, 2016; Gimeno et al., 2007; Qing, 2010; N. Sachindra & Mahendrakar, 2005; Sánchez-Camargo, Meireles, Ferreira, Saito, & Cabral, 2012).

1.3.4.1 Organic Solvents

Recovery of carotenoids (astaxanthin) from crustacean species have also been done using acetone, ethanol, methanol, isopropyl alcohol and ethyl acetate. N. Sachindra, Bhaskar, and Mahendrakar (2006) found that, compared to acetone, ethanol, isopropyl, methanol, petroleum ether, and ethyl acetate, a 50:50 mixture of isopropyl alcohol and hexane gave the highest carotenoid extraction yield (43.9 µg/g waste).

Extraction of astaxanthin with the use of solvents include supercritical (SC) fluid extraction (SFE) using carbon dioxide (CO₂) and a co-solvent, like an organic solvent (i.e. ethanol). This extraction technique offers several advantages including low temperatures, low pH to stabilize astaxanthin, and minimal to no oxygen and light exposure to cause oxidative or thermal deterioration (Charest, Balaban, Marshall, & Cornell, 2001; Torrissen, Tidemann, Hansen, & Raa, 1981). Studies using SC-CO₂ with ethanol, showed a significant increase in the extraction yield astaxanthin compared to SC- CO₂ without ethanol (Charest et al., 2001; Nobre et al., 2006; Sánchez-Camargo et al., 2012).

1.3.4.2 Acids and Enzymes

Depending on the condition for extracting carotenoids, some techniques have resulted in 50% losses of the carotenoid. To lessen the deleterious effects of certain techniques, pretreatment steps are suitable to increase the efficacy of extraction methods. Such pretreatment alters the matrix that carotenoids may be bound to. Barzana et al. (2002) use of enzymes to extract carotenoids from marigold flowers (*Tagetes erecta*) eliminated silage and drying processes that

resulted in considerable degradation of lutein, the predominate carotenoid. As mentioned before natural carotenoids are found in crustaceans as a carotenoprotein (protein-pigment) complex. Separating the carotenoprotein complex with enzymatic pretreatment prior to solvent extraction yielded a greater concentration of astaxanthin in hydrolyzed (38.91 $\mu\text{g/g}$) samples, compared to nonhydrolyzed (27.89 $\mu\text{g/g}$) samples (Armenta-Lopez 2002).

1.3.4.3 Edible Oils

Astaxanthin is a lipid soluble pigment, thus, many edible oils have been used as solvents to extract this pigment from crustaceans and fish byproducts (H. M. Chen & Meyers, 1984; Shahidi & Synowiecki, 1991). Such edible oils are mainly vegetable origin. N. Sachindra and Mahendrakar (2005) used different vegetable oils such as groundnut oil, sunflower oil, gingerly oil, coconut oil, rice bran oil, and mustard oil to compare extraction yields of carotenoids from shrimp byproducts and concluded that refined sunflower oil gave the highest extraction yield. In a study by Pu, Bechtel, and Sathivel (2010), flaxseed oil was used to extract astaxanthin from shrimp byproducts. It was noted that astaxanthin lowered the oxidation rate of the flaxseed oil.

Additionally, (Krichnavaruk, Shotipruk, Goto, & Pavasant, 2008) SC- CO₂ extraction of astaxanthin from *H. pluvialis* was done with the soybean oil and olive oil. The highest extraction efficiency obtained, of $36.36 \pm 0.79\%$ and $51.03 \pm 1.08\%$, respectively, was obtained and was far greater than what was extracted with pure SC- CO₂ ($25.4 \pm 0.79\%$).

The major pigments in oil extract are monoesters and diesters, suggesting that these esters are preferentially extracted by the oils. The extracted, esterified astaxanthins are considered to be less prone to oxidation than free astaxanthin (Omara-Alwala, Chen, Ito, Simpson, & Meyers, 1985). Free astaxanthin is either esterified with one or two fatty acids (FAs), or conjugated to proteins (carotenoprotein complex), such as in salmon and mussel muscle or snow crab and

lobster exoskeleton (Guerin et al., 2003; Henmi, Hata, & Hata, 1989; Manu-Tawiah & Haard, 1987; Ya et al., 1991; H.-P. Yang, Lee, Kim, & Kim, 1994)

1.3.4.4 Ultrasound Assisted Extraction

The major markets of astaxanthin sourced from *H. pluvialis* are nutraceutical and aquaculture markets. Inexpensive and less harsh physicochemical and cell disruption extraction methods (reduced toxicity and contamination from chemicals/solvents, and reduced structural changes by thermal treatments) are needed to yield increased amounts of naturally derived bioactive astaxanthin.

Novel extraction techniques have received a great deal of attention to overcome drawbacks of conventional extraction techniques. Drawbacks of conventional methods include the use of large amounts of solvents, exposure to elevated temperatures, lower extraction yields and prolonged extraction times. Novel techniques include supercritical carbon dioxide (SC- CO₂) extraction, microwave assisted extraction, and ultrasound assisted extraction (UAE). UAE has been used to extract and detect compounds in animals' structures and plant tissues (Kadam, Tiwari, & O'Donnell, 2013; Nessa, Khan, & Al Khatib, 2010; Vinatoru, Mason, & Calinescu, 2017; Yue, Xu, Prinyawiwatkul, & King, 2006).

Several proposed theories exist for explaining the extracting enhancement of UAE (F. Chemat et al., 2017). For extraction of astaxanthin from *H. pluvialis*, the theory of operation may be described as a detexturation effect. Detexturation of plant structures were noticed in a study on essential oil extraction by S. Chemat, Lagha, AitAmar, Bartels, and Chemat (2004). In their study, scanning electron micrographs showed gradual physical alterations (degradation of cell walls) with increasing processing times, between conventional extracted and UAE of caraway seeds used as a model compound. Such characteristic cell wall degradation favors accessibility of

the solvent to the intracellular components, rather than relying on diffusion alone, as in conventional extraction techniques. In a separated study modeling the extraction of astaxanthin, UAE improved the extraction efficiency of astaxanthin, when chemical and mechanical cell disruptions techniques were compared (D. Singh et al., 2015).

1.4 Lipid Digestion and Absorption

The gastrointestinal (GI) system, Figure 1.7, is responsible for the digestion and absorption of lipids. There are specific processes involved in the digestion and absorption of dietary lipids through the water-soluble environment of the GI tract (Lichtenstein & Jones, 2012). Dietary lipids (TG, phospholipids, steroids, and fat-soluble bioactive compounds) may be liquid or solid depending on temperature, and are referred as fats and oils, respectively. The bulk of dietary lipids are TG, which consist of a glycerol molecule esterified to three FAs.

1.4.1 Digestion

The first step of digestion begins in the mouth with salivation and mastication. Lohse et al (2007) suggested lingual lipase, produced by von Ebner's glands, starts the hydrolysis of free FAs from triacylglycerols (TAGs). TAGs are cleaved at the sn-3 position by lingual lipase, with increased efficiency towards short-chained FAs (Carey, Small, & Bliss, 1983). Carriere et al. (1993) reported that in the stomach, hydrolysis of TAGs continues with gastric lipase, secreted by chief cells of the gastric mucosa. Gastric lipase also cleaves TAG at the sn-3 position (Lichtenstein & Jones, 2012). The fat entering the upper duodenum is approximately 70% TG while the remainder is a mixture of partially digested hydrolysis products known as chyme.

The small intestine, the site where the majority of lipid digestion and absorption occurs, requires the actions of bile salts and pancreatic lipase, the principle enzyme involved in TAG digestion, which hydrolyzes ester bonds at the sn-1 and sn-3 positions (Canaan, Roussel, Verger,

& Cambillau, 1999; J.H.Peter & Rideout, 2010; Mu & Høy, 2004). Bile salts, phospholipids, and sterols are all lipid components of bile. Bile, an emulsifying fluid, is secreted directly from the liver and stored in the gall bladder in response to the presence of fat in the duodenum (Lichtenstein & Jones, 2012). The gall bladder also secretes pancreatic lipase. During digestion, lipids dissolve hydrophobic compounds and cause an increase in the secretion of bile from the gall bladder, which subsequently leads to the micellization of lipids (Hughes et al., 2009). Dietary mixed micelles are formed in the small intestine by 2-monoacylglycerol and free FAs (hydrolytic products of TAG), bile salts, phospholipids, unesterified cholesterol, and other fat-soluble substances (Salvia-Trujillo et al., 2017). Subsequently, the decreased size of the formed micelles and increased surface area over larger droplets aids in further solubilization and digestion of carotenoids (Furr & Clark, 1997).

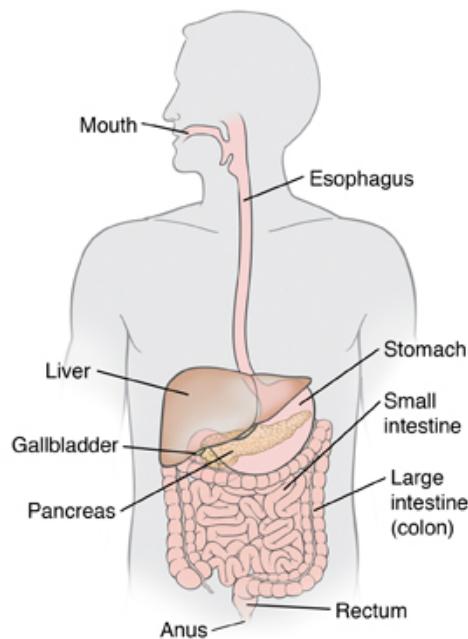


Figure 1.7. Gastrointestinal system anatomy. Source: <https://www.saintlukeskc.org/health-library/anatomy-digestive-system>.

1.4.2 Absorption of Carotenoids: Astaxanthin

A number of factors, described by the mnemonic “SLAMENGHI”, affect the absorption of carotenoids. *Species*, *Linkage*, *Amount* of carotenoid consumed in a meal, *Matrix*, *Effectors*, *Nutrient status*, *Genetic factors*, *Host-related factors*, and *Interactions* (Castenmiller & West, 1998; Lindshield, 2012). *Species* refers to the carotenoid being a xanthophyll or carotene, and relates to its geometric isomerism (BeMiller, 1986). The molecular linkage, whether free or esterified to FAs, is important for absorption also. The matrix refers to the accessibility of the carotenoids, being unbound or bound, becoming solubilized in micelles within the GI tract (Lindshield, 2012). It is critical for absorption to free carotenoids from bound matrices to improve uptake and increase bioavailability. Many effectors may contribute to the efficient absorption and bioconversion of carotenoids.

Diets consisting of many fruits and vegetables delay the onset of many age-related diseases, and contain a complex mixture of antioxidants (ascorbic acid, vitamin E, flavonoids, and carotenoids) (Everitt et al., 2006; S. Wang, Melnyk, Tsao, & Marcone, 2011). Since carotenoids are lipi-soluble, their absorption follows that of dietary fats. Additionally, intestinal absorption of carotenoids involves several critical steps: 1) release from the food matrix, 2) solubilization into mixed micelles in the lumen, 3) cellular uptake by intestinal mucosal cells, 4) incorporation into chylomicrons and 5) secretion of carotenoids and their metabolites associated into the lymph (E. H. Harrison, 2012). A number of factors affect the absorption of carotenoids, like species, linkage, amount, matrix, effectors, nutrient status, genetic factors, host-related factors, and interactions (Lindshield, 2012). *Species* refers to the carotenoid belonging to a group, xanthophyll or carotene, and relates to its geometric isomerization. However, the bioavailability of carotenoids is low, and varies from more than 50% in oily solutions to less than

10% from raw uncooked vegetables (Hinnant, Oh, Caburnay, & Kreuter, 2011; Olson, 1999). This low bioavailability reduces the distribution within the body by lowering the efficacy and potency of the carotenoid's therapeutic effects. Between the two groups of carotenoids, xanthophylls are ordinarily more readily absorbed than carotenes in part due to their hydrophobic nature, allowing them to be incorporated into the outer portion of lipid micelles in the lumen preceding absorption (Mercke Odeberg, Lignell, Pettersson, & Höglund, 2003; Yeum & Russell, 2002).

Bioavailability of astaxanthin is also be dependent upon digestion, intestinal absorption, and delivery to tissues (Couëdelo et al., 2010). The molecular linkage, whether free or esterified to FAs, is important for absorption. The matrix refers to the accessibility of the carotenoids, being unbound or bound, becoming solubilized in micelles in the GI tract (Lindshield, 2012). Many effectors contribute to the efficient absorption of carotenoids. Bioavailability of carotenoids is affected by the presence, amount, and type of lipid in the digestive tract at the time of absorption (Hughes et al., 2009).

Distribution of carotenoids among lipoproteins varies between carotenes and xanthophylls. The hydrophobic carotenes are found predominately in low-density lipoproteins. Guerin et al. (2003), reviewed the steps of digestion, absorption and plasma transport of carotenoids in mammals and found that polar carotenoids, such as astaxanthin, are likely to be transported by low-density lipoproteins (LDLs) and high-density lipoproteins (HDLs). Humans (along with monkeys, gerbils, and ferrets, but excluding rats, and mice unless given very high doses) absorb a significant portion of intact carotenoids directly and circulate or accumulate them in their plasma, liver, and peripheral tissue (X.-D. Wang, 2012). Things like vitamin A status, protein deficiency, and iron status are also known to affect absorption efficiency. Host

illnesses like atrophic gastritis, the process of chronic inflammation of the stomach mucosa, decreases carotenoid uptake due to insufficient gastric acid secretion, which disturbs the formation and absorption of mixed micelles (Yeum & Russell, 2002).

During digestion, lipids dissolve hydrophobic compounds and increase bile secretion from the gall bladder, which in turn leads to their solubilization for micellarization (Hughes et al., 2009). Inefficient absorption of astaxanthin may be due to several factors; including astaxanthin being embodied in food matrices, dissolution limitations in the GI fluids (*E*-isomer), and a saturated capacity of incorporation into bile micelles (Parker, 1996). X.-D. Wang (2012) suggested that food processing that causes mechanical breakdown of the food matrix and subsequent release of carotenoids can improve intestinal absorption, as in boiling of crustaceans like shrimp and crawfish.

1.5 Flaxseed Oil

In addition to the many health protecting benefits that AX has to offer and the potential to use an otherwise discarded material which may present environmental concerns, it would be ideal to use crustacean processing byproduct material as a natural source. A simple food processing method or heating can cause the mechanical breakdown of the matrix and release of the carotenoid, AX, from the processing byproducts (X.-D. Wang, 2012). Additionally, since AX is a lipophilic compound it can be extracted from processing byproducts using many plant derived edible oils, such as flaxseed oil.

Flaxseed oil, cold pressed from flax (*Linum usitatissimum*), or linseed, has gained a lot of attention due to its exceptional nutrient profile and protective effects against cardiovascular disease, inflammation, and some cancers (Bloedon & Szapary, 2004; C. R. Harper, Edwards, DeFilipis, & Jacobson, 2006; Tzang et al., 2009). Flax is the richest plant source of α -linolenic

acid (ALA; C18:3n-3), an omega-3 (n3) FA and lignans (phytoestrogens) (Barceló-Coblijn et al., 2008; Goyal, Sharma, Upadhyay, Gill, & Sihag, 2014; Oomah & Agriculture, 2001).

The importance of fat, and its lack thereof to severely impact growth and reproductive development in experimental animals was demonstrated by Evans and Burr (1927), despite the administration of fat-soluble vitamins. The works of the Burrs led to the discovery that FAs were crucial to health, and a deficiency could often lead to death. ALA is considered an essential fatty acid (EFA), a term coined by Burr and Burr (1930) for those FAs not synthesized by mammals and for which deficiencies could be reversed by dietary FA addition. ALA is a precursor of the n-3 FA family, the other being linoleic acid (LA); neither can be synthesized, *in vivo* (Jeffcoat & James, 1984). *In vivo* synthesis cannot occur because mammals lack the desaturase enzymes required to synthesize double bonds at the n-3 and n-6 position of the carbon chain of FAs. Therefore n-3 and n-6 FAs must be obtained from the diet (Jeffcoat & James, 1984; J. Lee, Lee, Kang, & Park, 2016). ALA is also a precursor to the long chain (n-3) polyunsaturated FAs (PUFA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) (C. R. Harper et al., 2006).

Of the total lipid content in flaxseed, thirty percent is composed of 100% lipids, of which 53% are ALA, 17% LA, 19% oleic acid, 3% stearic acid, and 5% palmitic acid; all of which provide an excellent n-6: n-3 FA ratio of approximately 0.3:1 (Simopoulos, 2002).

Table 1.2. Fatty acid content of flaxseed oil

Fatty Acids	Percentage (%) (Range)
Palmitic acid (C16:0)	4.90-8.00
Stearic acid (C18:0)	2.24-4.59
Oleic acid (C18:1)	13.44-19.39
Linoleic acid (C18:2) (ω -6)	12.25-17.44
α -Linolenic acid (C18:3) (ω -3)	39.99-60.42

Sources: (Anwar, Zreen, Sultana, & Jamil, 2013; Bozan & Temelli, 2008; Choo, Birch, & Dufour, 2007a; Guimarães et al., 2013; Khattab & Zeitoun, 2013; Pradhan, Meda, Rout, Naik, & Dalai, 2010; Pu et al., 2010; Quispe-Condori, Saldaña, & Temelli, 2011; Z.-S. Zhang et al., 2008; Z.-S. Zhang, Wang, Li, Li, & Özkan, 2011).

Flaxseed oil is low in saturated FAs, moderate in monosaturated FAs, and rich in PUFAs (polyunsaturated fatty acids), with ALA being the major FA (Table 1.2) (Goyal et al., 2014). Flaxseed oil provides the highest amount of ALA (40-60 wt% of total FAs) among various vegetable sources (Couëdelo et al., 2010). In other words, The antioxidant properties of ALA, like cardio protection, and other beneficial health effects of ALA have been attributed to its precursor role in converting to EPA in the body (Rajaram, 2014).

1.6 Lipid Oxidation

The general term used to describe complex sequences of chemical changes that result from the interactions between lipids and oxygen is known as lipid oxidation, or autooxidation at times (Damodaran & Parkin, 2017). Lipid oxidation can either be mediated by enzymes (i.e. lipoxygenase, cytochrome P450s, and cyclooxygenases), nonenzymatic and nonradical photooxidation, or by nonenzymatic chain autooxidation pathways (i.e. reactions with oxidants and free radicals mentioned in the following paragraphs). Both enzymatic and non-enzymatic oxidation pathways are responsible for physio-pathological effects seen in cellular functions and the development of diseases (Figure 1.8). The last two types of reaction pathways consist of a combination of reactions involving triplet oxygen $^3\text{O}_2$.

PUFAs are more readily attacked by oxidants because of the high number of carbon-carbon double bonds contained within their structure (Ayala, Muñoz, & Argüelles, 2014). Additionally, the high susceptibility of lipids to oxidation is the reason why they are an excellent energy source for catabolic metabolism. Lipid oxidation of foods containing lipids during food processing and storage is of major concern because it can create undesirable qualities in a finished product. Lipid oxidation can be initiated (ensue the formation of free radicals) by a number of factors (initiators) including heat, UV and ionizing radiation, peroxides and trace metals (i.e. copper or iron ions), reaction (1):

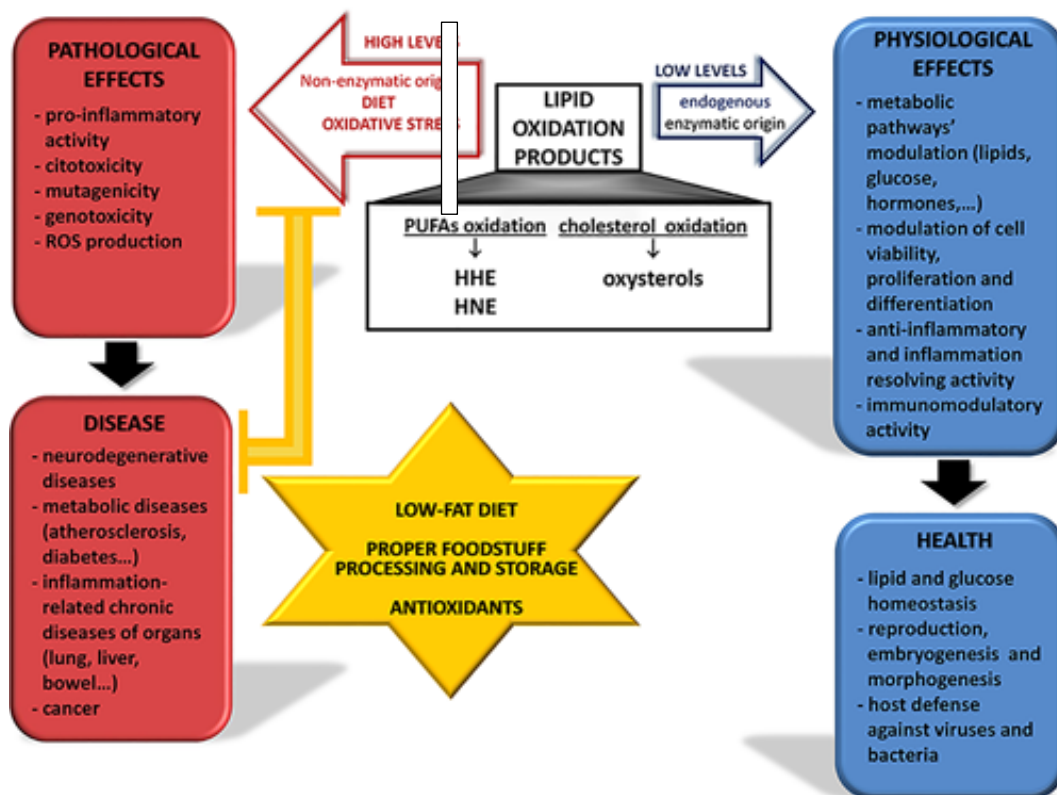


Figure 1.8. Lipid oxidation occurring through both enzymatic and non-enzymatic reactions. Source: Sottero et al. (2019)

During lipid oxidation, the hydroperoxides formed from unsaturated FAs like linoleic acid (LA; C18:2n-6) and arachidonic acid (AA; C20:4n-6) readily undergo autoxidation, the key mechanism in lipid oxidation, due to the ease of which a hydrogen atom can be abstracted from the molecules, forming a free radical. Meaning, the autoxidation rate increases with the degree of unsaturation in lipids. The processes of lipid oxidation follow the same order regardless of it being in an emulsion or other food product as it does in an oil, and are discussed in further detail. Nonenzymatic FA oxidation, also termed autooxidation, can be described by three distinct phases, known as initiation, propagation and termination.

1. Initiation

It is unlikely for the non-enzymatic unsaturated FA (LH) oxidation, reaction (2), to proceed spontaneously because of the high activation energy resulting from the spin barrier between lipids and $^3\text{O}_2$ (Laguerre, Lecomte, & Villeneuve, 2007). Therefore, it begins with the homolytic breakdown of hydrogen in the



During this step, peroxides are cleaved and formed into alkoxy and hydroxyl radicals and act as initiators of subsequent chain reactions (Halliwell & Chirico, 1993). During this initiation step, unsaturated FAs (chemical species) with unpaired electrons called the alkyl radical (L^*), are formed. Lipid oxidation is mostly initiated by hydroxyl ($^*\text{OH}$) and hydroperoxyl (HOO^*) radicals (reaction (3)). Additionally, lipid alkoxy (LO^*) and peroxy (LOO^*) radicals (reaction (4)) can act as initiators as well. Hydroxyl radicals have the highest reaction rate with lipids; thus, the importance of their reduction is crucial to oxidative stability. Antioxidants can counteract lipid oxidation by protecting FAs from oxidation initiators or by stalling the second phase, propagation (intercepting radical oxidation *propagators*).



2. Propagation

The propagation phase is primarily a radical chain reaction. During this phase, peroxide radicals react with oxygen to form a peroxy radical (LOO^*) which potentially accelerates lipid degradation due to its high energy (reaction (5)). Hydrogen addition to the peroxy radical results in the formation of hydroperoxides (LOOH) and a new L^* , the primary oxidation compounds that can react with other radicals to form volatile compounds (reaction (6)). In order to slow down this reaction, the oxygen level should be very low, or an antioxidant can be used to intercept lipid peroxy (LOO^*) radicals, a radical oxidation propagator (Laguerre et al., 2007).



3. Termination

The propagation phase persists with the transformation (decomposition) of hydroperoxides into secondary nonradical compounds. The termination reactions leads to the formation of hydrocarbons (dimers), volatile and nonvolatile ketones, alcohols, and oxidized triacylglycerols and polymers (Halliwell & Chirico, 1993; Laguerre et al., 2007). When polyunsaturated FAs oxidize and become alkyl radicals (R^*), they form FA dimers (R-R) and hydroperoxide dimers (R-OO-OO-R), the major initial oxidation products susceptible to further oxidation or decomposition to secondary reaction products (i.e. short-chain aldehydes, dimers (reaction (6)), ketones (reaction (7)) and other oxygenated (non- radical) compounds (reaction (8))). A single polyunsaturated hydroperoxide radical (ROO^*), primary oxidation compound, can generate several types of volatile or nonvolatile molecules.



The secondary reaction products may undesirably affect the overall quality of foods, including flavor, odor, nutritional value and the production of toxic compounds that cause diseases, especially within anaerobic environments, i.e., frying oils (Spickett et al., 2015; Vercellotti, St Angelo, & Spanier, 1992).

1.7 Hydrocolloids

Colloids (e.g., proteins, starches, and fats), never truly dissolve in a solvent, but remain in an unstable colloidal dispersion. These dispersions contain particles larger than molecules, yet too small to be seen. Hydrocolloids are colloids with an affinity for water. as defined by the etymology of the word. Hydrocolloid, of Greek origin with *hydro* meaning ‘water’ and *kolla* meaning ‘glue’ (Wüstenberg, 2015). Natural hydrocolloids are biopolymers that originate from living organisms. Additionally, hydrocolloids will increase the viscosity an aqueous system and all will bind and hold water to different extents. The two main uses of hydrocolloids are thickening agents in soups, gravies, and sauces and the other use is as a gelling agent is jams, jellies and low sugar gels (Saha & Bhattacharya, 2010). A gel is defined as a largely diluted system that exhibits no steady flow (Ferry, 1980). Gels are typically made of polymers cross-linked by covalent or non-covalent interactions to form a network capable of entrapping water and low molecular weight materials. Simply stated, a gel is a transitional phase between a solid and a liquid. A few other functional properties include film formation, encapsulation, and improving shelf-life (Paulomi Burey, Bhandari, Howes, & Gidley, 2008; Dickinson, 2003, 2009).

A secondary function of hydrocolloids is reducing the flavor intensity by increasing a food products thickness. These are all important factors when formulating a food product or a delivery system. Even though most hydrocolloids are polysaccharides from natural sources like pectin from citrus peels and apple pomace, and carrageenan from seaweed, some are proteins, like gelatin from collagen from animals, and gliadin from wheat (BeMiller, 2008; Paulomi Burey et al., 2008; Endress & Christensen, 2009; Mariod & Fadul, 2013; X. Wang, Chen, & Lü, 2014).

The hydrocolloids gelatin and pectin are two biopolymers capable of forming a gel, the in-between phase amid a solid and a liquid. A gel is defined as a largely diluted system that exhibits no steady flow (Ferry, 1980). Simply stated, a gel is a transitional phase between a solid and a liquid. Gels can be made up of polymers cross-linked by covalent or non-covalent interactions to form a network capable of entrapping water and low molecular weight materials. Gelation is defined as the phase transition from a state without a gel to a state with a gel, that involves the formation of an “infinite” network (Stauffer, Coniglio, & Adam, 1982).

1.7.1 Gelatin

Gelatin, Figure 1.9 is obtained from the partial hydrolysis of the polypeptide backbone of collagen from pigskin, cattle bones and cattle hide (46%, 29.4%, 23.1% of total production, respectively) (Duconseille, Astruc, Quintana, Meersman, & Sante-Lhoutellier, 2015; Karim & Bhat, 2008). The name gelatin is derived from the Latin *gelata*, describing the most characteristic quality, i.e. gel formation in water (Ledward, 2000). First used as a glue as far back as 600 B.C., gelatin, a biopolymer made of water-soluble protein (85-92%), belonging to a unique class of hydrocolloids that have many functions and applications, is now used as a food and pharmaceutical ingredient for its gelling and foaming ability (Duconseille et al., 2015; Karim & Bhat, 2008). Gelatin is also an essential ingredient in the preparation of a confectionery with a

hard, chewy or foamy texture. There are two types of gelatin, type A from acid (mineral acid, pH 1.5 to 3.0) pretreatment, and type B from alkaline (saturated lime, pH 12.0) pretreatment. In the pharmaceutical industry gelatin is the main ingredient used in making hard capsules. A key feature of such capsules is that they melt at temperatures above 30 °C enabling them to easily release encapsulated drugs they contain in the human digestive tract. In addition to gelatin possessing the ability to form a gel, they can act as emulsifiers and stabilizers of colloid systems.

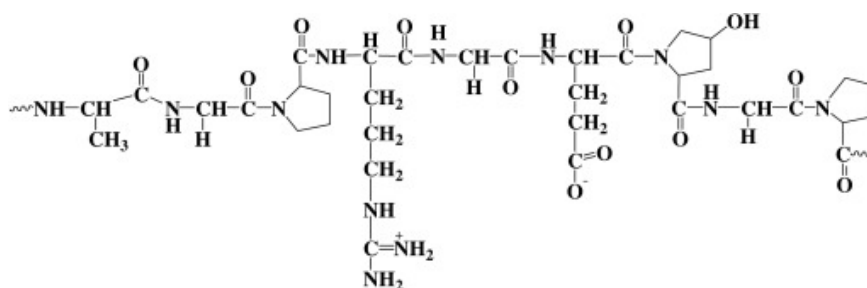


Figure 1.9. Chemical structure of gelatin.

1.7.2 Hydroxypropyl methylcellulose (HPMC)

An alternative to gelatin, hydroxypropyl methylcellulose (HPMC, Figure 1.10) was developed in the industry from other polymers in an attempt to replace gelatin due to regulatory concerns and drug incompatibility (Al-Tabakha, 2010; Duconseille et al., 2015). HPMC is a synthetic modification of the natural polymer cellulose, the most common organic compound and biopolymer on earth. HPMC has been used as a thickening agent, as a coating polymer, in controlled release systems, and even in solid dispersions to enhance drug solubility (C. L. Li, Martini, Ford, & Roberts, 2005). A major defining difference between biopolymers and synthetic polymers can be found in their structures.

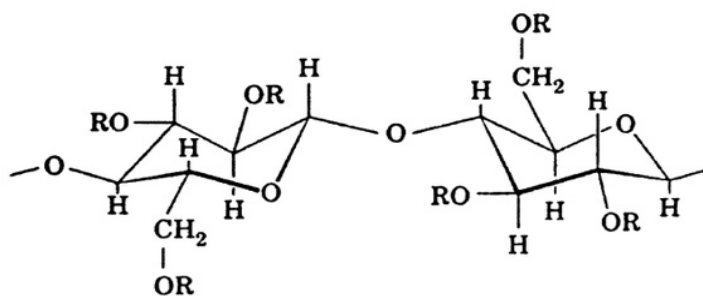


Figure 1.10. Chemical structure of hydroxypropyl methylcellulose (HPMC). Source: Phadtare, Phadtare, Nilesh, and Asawat (2014)

1.7.3 Pectin

Pectin, a biopolymer with stabilizing and gel forming capabilities, is an anionic polysaccharide complex of branched heteropolysaccharides from the middle lamella and cell walls of plants, consisting of partially esterified galacturonic acid residues, see Figure 1.11 (Baciu & Jördening, 2004). Pectin for the commercial industry is mainly derived from apple pomace and citrus peel, byproducts from juice manufacturing. Even though pectin's are found most commonly in terrestrial plant tissues and cell walls, they do not all possess the ability to form gels. The gel forming ability of pectin depends on the molecular weight and the degree of methylation (DM) (Thakur, Singh, Handa, & Rao, 1997). The DM is particularly important when considering the formulation of a drug delivery system (Ashford, Fell, Attwood, Sharma, & Woodhead, 1994), that targets specific sites within the digestive system. Pectins with low degrees of methylation (LM pectin), DE < 50%, are relatively soluble but can be cross-linked by divalent cations (calcium, Ca) to produce insoluble gels. Conversely, pectins with high degrees of methylation (HM pectin), DE > 50%, are poorly soluble, and gel with high concentrations of sugar (Löfgren & Hermansson, 2007; Rolin, 1993). Additionally, pectin can be used to encapsulate bioactive compounds.

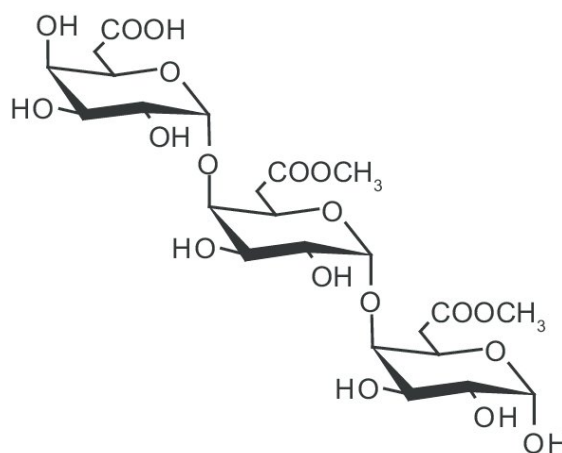


Figure 1.11. Structure of pectin. Alpha 1, 4 linked D-galacturonic acid. Source: Ali, El-Regal, and Saeed (2015)

1.7.4 Interactions of protein and polysaccharides

There are multiple interactions known to occur between proteins and polysaccharides. These specific and non-specific interactions cannot be fully understood without prior research, and are critical in formulations. Interactions of polysaccharides and protein range from complete segregation to complexation (Figure 1.12). Both proteins and polysaccharides are present together in many food systems, contributing to the structure, texture, and stability of the food independently, through their surface properties and distinctive thickening or gelling behavior. Albeit the vast information known at the molecular level about the functionality of individual biopolymers, our knowledge of the role of protein-polysaccharide interactions, as it relates to their functionality in emulsions, gels or food mixed solutions is somewhat limited.

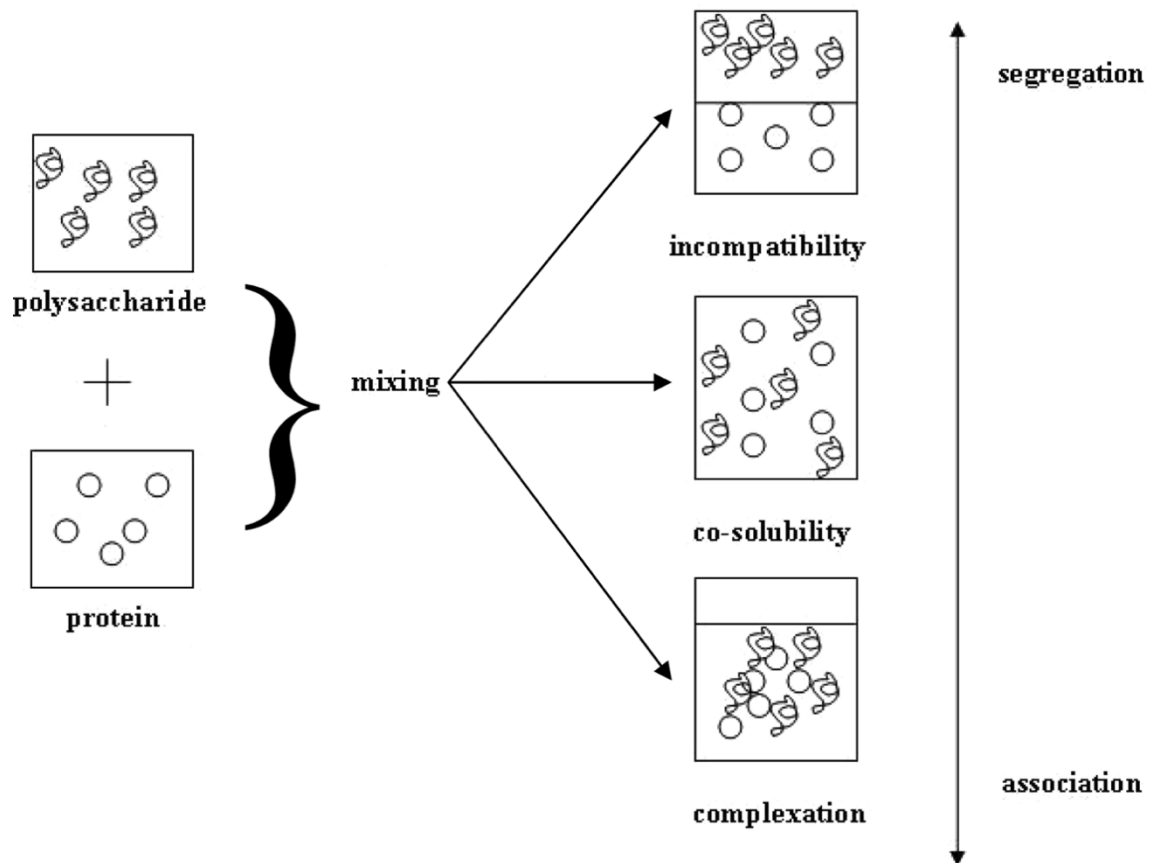


Figure 1.12. Main trends in the behavior of biopolymer mixtures. Source: P. Burey, Bhandari, Rutgers, Halley, and Torley (2009).

Biopolymer interactions in food systems are typically thermodynamically characterized by separating them into enthalpy or entropy driven types. Properties of food products, i.e., flow, stability, texture, and mouthfeel, are all influenced by protein-polysaccharide interactions in the aforementioned thermodynamic categories. When mixing two biopolymers in solution, one of the three possibilities depicted in Figure 1.12 may be observed. The resulting interactions will either be segregative or associative. However, in very dilute solutions the proteins and polysaccharides are co-soluble. Conversely, upon increasing the concentrations of the biopolymers, the co-solubility may be lost. Biopolymer mixtures tend to segregate more often than not (de Kruif & Tuinier, 2001).

The association between protein and polysaccharide molecules can occur chemically

through covalent bonds or physically through electrostatic interactions. Attraction and repulsion are the two major types of interactions that occur between proteins and polysaccharides in solution that may result in complex formations (coacervation), co-solubility or immiscibility of the two biopolymers as seen in Fig. (Benichou, Aserin, & Garti, 2002). The electrostatic attraction between pectin and gelatin leads to complexation and coacervation.

Bungenberg de Jong (1949) first described the formation of coacervates and complex coacervates. In general, coacervates are formed reversibly. The word coacervate was used because the complex remained liquid, rather than precipitated. This is a phenomenon that occurs quite often in biopolymer mixtures in which the biopolymers are oppositely charged.

Many polysaccharides carry anionic groups; pectin and gum arabic (GA), for instance, carry carboxylic groups. Pectin contains anionic groups (carboxylic groups) in its structure that are negatively charged, while other polysaccharides contain phosphate and sulphate groups. Gelatin, being an ampholytic protein, can form a complex coacervate with polysaccharides. Gelatin being ampholytic means it is anionic at $\text{pH} > \text{pI}$ and cationic if $\text{pH} < \text{pI}$, where pI is the iso-electric pH, defined as the pH where the net charge is zero. Gelatin also carries many lysine groups and can therefore be used to form complexes with anionic polysaccharides such as pectin. A complexation of this type is used in encapsulation. For instance, GA is adsorbed onto an oil–water interface together with gelatin, thus ‘coating’ the oil droplet with both biopolymers. In this way one can use biopolymers as emulsifier although they do not adsorb onto emulsion droplets without the other biopolymer.

Like many other carotenoids, being non-polar and lipid-soluble, the absorption of astaxanthin is enhanced with dietary oils (Ambati et al., 2014; Erdman, Bierer, & Gugger, 1993; Fernández-García et al., 2012; Parker, 1996). Considering the previously discussed factors

affecting the absorption and bioavailability of astaxanthin, it is important to consider a delivery system to improve upon those affected processes. The metabolic fate of bioactive compounds *in vivo* does not depend solely on intrinsic factors; however, it is more determined by the carrier system. Based on human physiology and the mechanism of lipid absorption, the incorporation of such astaxanthin into a delivery system for intestinal uptake and absorption would be beneficial.

In efforts to overcome the susceptibility of bioactive compounds to a loss of function or destruction and improve their stability during processing and storage, emerging technologies of nano-/micro-encapsulation are applied in food and nutraceutical industries. Besides protecting them from the harsh processing conditions and adverse storage environment, the encapsulations of bioactive compounds can also achieve targeted delivery and controlled release of entrapped nutrients to the specific site. Encapsulation is a technique employed to create functional foods, guaranteeing the stability of functional constituents while allowing their application to diverse foods (Dias, Botrel, Fernandes, & Borges, 2017). Encapsulation is a process that creates a barrier over the active component that protects against the effect of environmental factors and allows the controlled release of the active component under certain conditions. Different delivery systems including liposomes, emulsion and complex coacervates, are appropriate to create protective barriers. However, complex coacervates, were chosen as the preferred delivery system due to its key advantages of heat resistance and a higher loading capacity (Muhoza, Xia, Cai, et al., 2019).

The controlled release of bioactive compounds is needed to prevent such compounds from being completely solubilized and immediately bioavailable following oral ingestion. To accompany the immediate release of bioactive compounds after oral ingestion, a characteristic pattern featuring high plasma concentrations of the compound shortly after ingestion ensues. This is followed by a sharp decrease, as it is expeditiously absorbed into the bloodstream and

cleared from the system (Turner et al., 2004).

Both increased free radical production and reduced antioxidant defenses both lead to oxidative stress. Oxidative stress is defined as an imbalance between prooxidants (reactive oxygen species (ROS) and other reactive metabolites, i.e. reactive nitrogen species) and their elimination by antioxidants, which is in favor of the prooxidants (Reuter, Gupta, Chaturvedi, & Aggarwal, 2010; Sies, 2013). As a result of oxidative stress, the production of biomarkers (i.e. cytokines, protein carbonyls, and thiobarbituric acid reactive substances relating to lipid peroxidation) is increased via several different mechanisms (Elmarakby & Sullivan, 2012; J. Liu et al., 2000). The production of cytokines causes a cascade of additional cytokines to be secreted, ultimately leading to deleterious effects on the body. Exercise increases rates of oxygen consumption by the whole body and tissue causing oxidative stress and tissue damage (J. Liu et al., 2000). However, this damaging response is attenuated if exercise is done repeatedly as the tissue acclimates to the new overload stress

Studies suggest the addition of an antioxidant is required to achieve an overall positive effect of dietary oils, as it relates to the further oxidation of components within the oil that potentially lead to increased oxidative damage and shortening of the lifespan in animal models (Tsuduki, Honma, Nakagawa, Ikeda, & Miyazawa, 2011). There is a synergistic effect between AX and edible oils, for example AX may offer protection to the oil from oxidation and the oil improves the stability and absorption of the AX. Studies have reported that carotenoids are absorbed in the body like lipids and undergo the same transport mechanisms as does lipids. Nonetheless, flaxseed oil is used to extract AX in order to create a carotenoid rich edible oil (FOAX) that can be used in further processing steps to add nutritive value. The extraction of AX using (flaxseed oil), to assess the effect of FO and AX together against oxidative stress as well as

the synergy between the two, relating to slowing the oxidation of the FO and subsequently creating a delivery system with the flaxseed oil (FO) containing AX (FOAX) to investigate the transport of astaxanthin for absorption.

1.8 Cancer trends and treatment

Cancer, a collection of related diseases, impacts the lives of many people all around the world. Cancer, occurring among men and women alike, can start almost anywhere in the body (see Table 1.3). Recently it was projected for 2019 that 1,735,350 new cancer cases would occur in the United States (Siegel, Miller, & Jemal, 2019). Of those projected new cases, 34.4% are a result of patient mortality due to the disease. Cancer is the second leading cause of death worldwide. With normal human physiological processes, cells grow and divide to form new cells as the body requires. Those new cells take the place of old or damaged cells. Conversely, with cancer the body's cells divide without stopping, regardless of being damaged, and spread into surrounding tissues potentially causing tumors.

Table 1.3. Percentile of the Projected (1,762,450) New Cancer Cases and Survival Rates

Cancer sites (part)	% of projected new	
	cases	% Survival
Oral	3	79.5
Breast	15.4	84.4
Digestive system	18.6	
Esophagus	1	8.9
Stomach	1.5	59.5
	5.8	49.7
Liver	2.4	24.4
Pancreas	3.2	19.4
Respiratory system	13.9	
Lung	12.9	37.4
Skin	5.9	
Melanoma	5.4	92.5
(table cont'd)		

Cancer sites (part)	% of projected new cases	% Survival
Genital system		
Ovaries	1.3	37.9
Prostate	9.9	81.9
Brain	1.4	25
Lymphoma	4.7	74.5
Leukemia	3.5	63

Source: Siegel et al. (2019)

Tumors can be either benign or malignant. As a malignant tumor, capable of spreading into or invading neighboring tissues, some of the cancer cells may disassociate and travel to other sites in the body through the blood or lymphatic system and form new cancerous tumors (metastasizing). On the other hand, benign tumors do not spread or invade adjacent tissues. Albeit benign tumors do not grow back after removal, some can be life threatening, like benign brain tumors (Hill, Nixon, Ruehmeier, & Wolf, 2002).

Genes are fragile and a variety of things can damage them, including sunlight and how they are copied during proliferation. In chromosomal DNA only 5 to 10 percent of all cancers are genetic in origin. According to Glaire and Church (2019), cancer, a disease of a disordered genome, is an unregulated, disproportionate division of cells. This is recognized pathologically by a greater proportion of cells actively in the cell cycle than is ordinarily present in the normal tissue from which it originated. Meaning a higher fraction of cells are in mitosis, recognizable by microscopy, and a higher fraction of cells are in S-phase, where DNA replication occurs.

1.8.1 Breast Cancer

Of the 100 or so different types of cancer, breast cancer, the most common form of cancer in women globally, is the second leading cause of deaths for women in the United States (Boyle, 2012). About 12% of women in the United States will develop invasive breast cancer over the course of their lifetime (Brocker, 2017; Friedman, 2018; Gemignani, 2016; J. Harrison,

2019; Hill et al., 2002). Breast cancer comprises distinct subtypes that have different biological and clinical behaviors. Researchers have defined four main classical subtypes of breast cancer based on biological and clinical meaningfulness pertaining to how abnormal the cancer cells and tissue look microscopically and how quick they are likely to metastasize (Weigelt, Geyer, & Reis-Filho, 2010). The subtypes differ by their expression of the estrogen receptor (ER), progesterone receptor (PR) and the human epidermal growth factor receptor 2 (HER2 positive) (Tyanova et al., 2016).

ERs are nuclear receptors belonging to the steroid hormone receptor superfamily that mediate the effects of the C₁₈ sex steroid, 17 β -estradiol (E2), produced by the testis and ovaries in both men and women, respectively (Enmark & Gustafsson, 1999; Hess et al., 1997). E2 has primary effects on females during their reproductive and development years, while also playing a critical role in male sexual function (Brock, Baum, & Bakker, 2011; Schulster, Bernie, & Ramasamy, 2016). Darbre, Yates, Curtis, and King (1983) found that E2 increased the growth rate of a human breast cancer cell line that is positive for ER expression. Similarly, a second nuclear receptor belonging to the steroid hormone receptor superfamily, known as PR, mediates the effects of the C₂₁ sex steroid, progesterone (Scarpin, Graham, Mote, & Clarke, 2009). Progesterone is critical for the normal reproductive function of women, and has been shown to increase the proliferation of luminal epithelial cells (Clarke, Howell, Potten, & Anderson, 1997; Masters, Drife, & Scarisbrick, 1977; Meyer, 1977; Potten et al., 1988). Cells that are ER/PR-positive are much more likely to respond to hormone therapy than those that are ER/PR-negative. HER2, a member of the epidermal growth factor family, when overexpressed is associated with a more aggressive, decreased survival, and higher recurring form of disease (Mitri, Constantine, & O'Regan, 2012). Lastly, there is a subtype known as, triple-negative

Figure 1.13. The triple-negative breast cancer (TNBC) cells are so termed because they lack expression of the ER, PR, and HER2 receptors. TNBC cells present a poor prognosis and are considered to be more aggressive and the most difficult to treat due to there being fewer targeted medicines available.

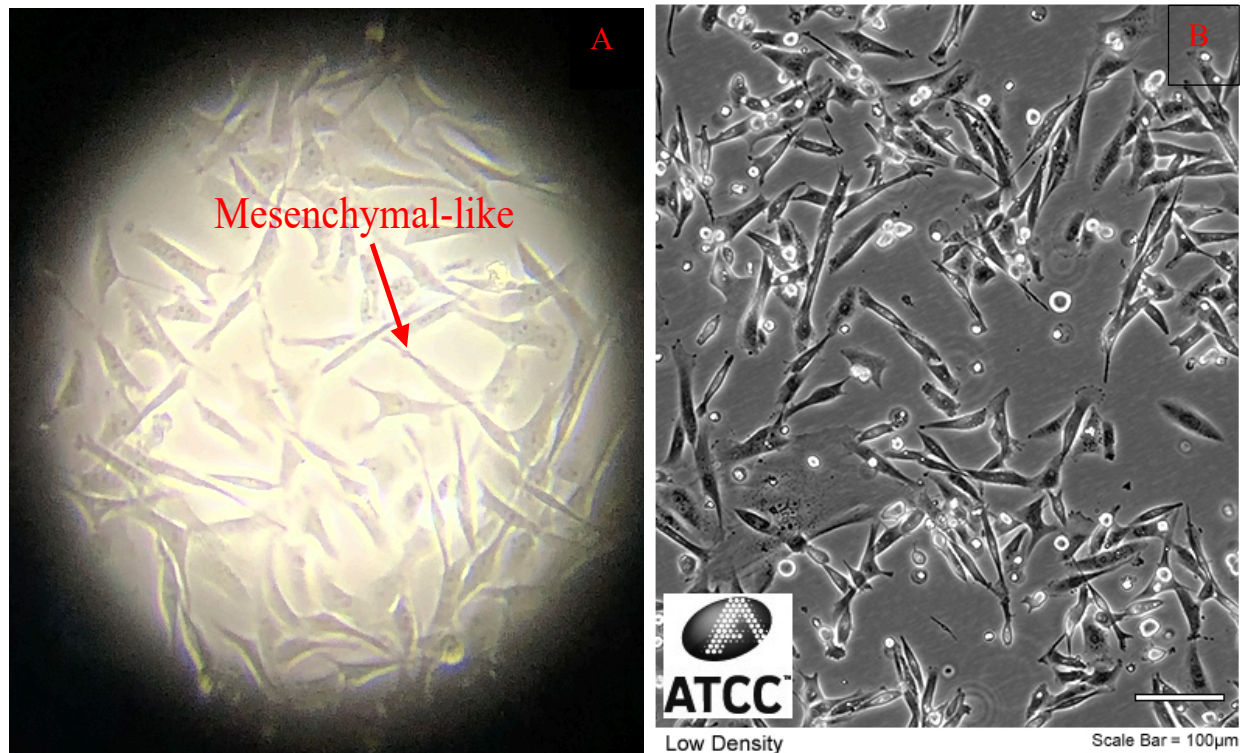


Figure 1.13. (A) MDA-MB-231 epithelial cells expressing mesenchymal-like phenotypes (high number after 2 days incubation in DMEM at 37 °C). (B) MDA-MB-231 (ATCC® HTB-26™) epithelial cells expressing mesenchymal-like phenotypes. Source: ATCC® (2020)

1.8.2 Breast cancer treatment

In the event that cancer develops, the key to successful treatment is early detection.

Prompt early diagnostic and accessible treatment for cancer is critical for survival (Organization, 2017). Cancers are treated with a combination of drugs (angiogenesis inhibitors, modulation of signaling pathways), chemotherapy (kill or stop proliferation), radiation (kill or stop proliferation), and/or surgery (removal of tissue infected with cancerous cells)(Arora & Scholar,

2005; Lebert, Lester, Powell, Seal, & McCarthy, 2018).

Since the dawn on the 21st century, substantial advancements have been made in the treatment of hormone responsive tumors and gene amplified subtypes, ER+ and HER2+, respectively, with various types of therapies available including monoclonal-antibody based and hormonal therapies and small molecule inhibitors of tyrosine kinases for both localized and metastatic forms of cancer (L. Chen, Linden, Anderson, & Li, 2014; W. Y. Chen et al., 2004; Ma, Bernstein, Pike, & Ursin, 2006; Verkooijen, Bouchardy, Vinh-Hung, Rapiti, & Hartman, 2009; T. Yoshida et al., 2014). As a result, survival rates of patients diagnosed with the luminal or amplified subtypes have improved tremendously, particularly for those cancers detected early. However, the distinct phenotypes, molecular profiles, and lack of viable receptors for targeted therapy of TNBC result in the ineffectiveness of therapies recommended for other subtypes of breast cancer.

TNBC present a major challenge for oncologist, as they comprise 10-15% of all breast cancers diagnosed with limited treatment. This subtype of cancer is more commonly seen in young and obese women, with a greater prevalence in premenopausal African American women (Fang et al., 2017; Schäfer et al., 2019). Only systemic treatment options, chemotherapy combined with standard cytotoxic agents, are available to patients with TNBC.

However, with systemic treatment options, comes great risks. Cytotoxic agents having a narrow therapeutic window combined with the complexity of their pharmacologic profile results in a higher risk of experiencing side effects. One problem that may be of subject to scrutiny is permanent bone marrow damage associated with cancer treatments (Lohrmann, 1984). While chemotherapy is a principal component of treatment for cancer, the damaged induced on healthy cells while damaging cancer cells leads to co-morbidity and sometimes mortality (Pearce et al.,

2017). Most side effects of chemotherapy affect the patient's quality of life, (i.e. appetite loss, thrombocytopenia, edema, delirium, diarrhea, constipation, fatigue) and the attempts to manage such side effects have decreased survival rates (Abrams et al., 2012; Blauwhoff-Buskermolen et al., 2016; Rutten et al., 2016).

1.8.3 Finding alternative treatment

Researchers focus on the genome and their RNA transcripts to develop alternative therapeutic routines in an effort to decrease the co-morbidity and mortality associated with treatment of TNBC. A model of TNBC is the MDA-MB-231 cell line, an immortalized human metastatic cell line isolated at The University of Texas MD Anderson Cancer Center from a pleural effusion of a patient with invasive ductal carcinoma (Welsh, 2013). MDA-MB-231 cells are ER-negative, PR-negative, HER2-negative, and express mutated p53.

Additionally, the MDA-MB-231 cell line is mesenchymal in phenotype and have repressed expression of epithelial (E)-cadherin to facilitate release from the tumor and gain N-cadherin to attach at distal sites. Cadherins are cell adhesion molecules (CAM) that function to bind cells to one another. Neural (N)-cadherin (CDH2), a cell adhesion molecule that can cause disassembly and cell migration when inappropriately expressed (Islam, Carey, Wolf, Wheelock, & Johnson, 1996). CAMs are important in cancer invasion and metastasis. During embryonic development, organ fibrosis and wound healing a biological process takes place where epithelial cells convert to a mesenchymal phenotype (Roche, 2018). After such conversion, epithelial cells lose their cell polarity and cell-cell adhesion, gaining migratory and invasive properties (fibroblast properties). This process is known as an epithelial-to-mesenchymal transition (EMT), and the resulting mesenchymal cells are capable of differentiating into a variety of cell types. Moreover, EMT is not complete in cancer cells, so they express both epithelial and mesenchymal

genes, making them more aggressive than cells with a complete EMT phenotype (Jolly et al., 2015). In cancer cells, an increase in N-cadherin, disruption of cell-cell adhesion, expression and a decrease in C-cadherin are characteristic of EMT.

Research has focused extensively on methods to inhibit or even reverse the EMT process and induce mesenchymal to epithelial transition (MET) to inhibit tumor metastasis. The anticancer properties of natural compounds are rapidly gaining interests of many researchers for this. Varied classes of natural compounds have demonstrated anticancer properties including marine-based bioactive compounds (Fan et al., 2018; Kim, 2014; Simmons, Andrianasolo, McPhail, Flatt, & Gerwick, 2005). Carotenoids are associated with numerous health benefits. One in particular, shown in many epidemiological studies, is the inverse relationship between various cancers and a high intake of carotenoid containing foods (Astorg, 1997; Martí, Roselló, & Cebolla-Cornejo, 2016; Ni, Yu, Wang, Zhang, & Shen, 2017; Nishino, Murakoshi, Tokuda, & Satomi, 2009; Rowles III & Erdman Jr, 2020). However, the exact mechanisms by which the protection is afforded is still under investigation. Furthermore, in addition to their antioxidant and provitamin A activity, carotenoids can affect cell growth, regulation, and modulate immune and gene expression (Mercke Odeberg et al., 2003).

CHAPTER 2 EXTRACTION OF CRAWFISH AND SHRIMP ASTAXANTHIN WITH FLAXSEED OIL: EFFECTS ON LIPID PEROXIDATION AND ASTAXANTHIN DEGRADATION RATES

2.1 Introduction

Louisiana is the nation's leader in production of crawfish (*Procambarus clarkii*) and in shrimp harvesting, whose seasons run from November to July (crawfish) or generally year-round (shrimp), and is reported to be the world's second-largest seafood producer by volume (Delcambre, 2018; Simon-Friedt et al., 2016). The crawfish industry of the South Louisiana, better known as the crawfish capital of the world, is a multi-million-dollar industry. Louisiana is also home to an excess of 90% of the crawfish farms in the United States.

As a result of crawfish processing (peeling), crawfish processing byproducts (CB) are generated containing cephalothorax, abdominal exoskeleton, and viscera, which accounts for approximately 85% of the whole crawfish (H.-M. Chen & S. P. Meyers, 1982). The processing byproducts generated from crustacean species are a good source of high-quality bioactive compounds, i.e. astaxanthin, and there are opportunities to use such processing byproducts to extract astaxanthin. Astaxanthin can be used as a natural antioxidant ingredient in foods, feeds, and nutraceutical applications. Astaxanthin, the main pigment found in salmonids and crustacean species provides the desirable reddish-orange color in many organisms (Additives et al., 2019).

Additionally, the antioxidant activity of astaxanthin is one of its most important properties. It has been reported that the antioxidant activity of astaxanthin is 10 times higher than other carotenoids such as zeaxanthin, lutein, canthaxantin, and β -carotene (Miki, 1991; Yuan, Du, Jin, & Xu, 2013). Several studies have identified astaxanthin as having antioxidant mechanisms that quench active oxygen species and free radicals *in vitro* and *in vivo* (Edge and others 1997; Palozza and Krinsky 1992; Rengel and others 2000). Due to its antioxidant

properties, astaxanthin may have a role in the treatment of chronic diseases such as cardiovascular diseases, cataract development, macular degeneration, and cancer (S. T. Mayne, 1996).

There has been an ever-growing increase in the potential markets for natural antioxidants in soft drinks, ice cream, desserts, candies, meat products and pet and aquaculture feeds (Delgado-Vargas and others 2000; Delgado-Vargas and Paredes-Lopez 2003). Although natural carotenoids only accounted for 24% of the world market in 2014, they are expected to grow an additional 3.9% in response to increasing consumer demands (Joel, 2015). Astaxanthin was valued at \$512.6 million in 2016, and is predicted to reach \$814.1 million by 2022 (Markets, 2017).

Astaxanthin can be extracted from crawfish and shrimp processing byproducts using solvents or edible oils. H. M. Chen and S. P. Meyers (1982) have extracted astaxanthin from crawfish processing byproducts using soybean oil. Since, studies have been done to further increase the biological function and stability of astaxanthin by extracting it with a more health beneficial edible oil. Astaxanthin has been investigated for its protective effects in flaxseed oil, which is used as an extraction solvent (Pu & Sathivel, 2011). An established extraction procedure for extracting astaxanthin from crawfish and shrimp processing byproducts was developed to microencapsulate flaxseed oil containing astaxanthin (Pu, Bankston, & Sathivel, 2011a, 2011b).

Antioxidant-rich natural astaxanthin dispersed in alpha linolenic acid-rich flaxseed oil may provide a healthier functional food options for US consumer. Flaxseed oil has high content of alpha-linolenic acid (ALA) and linoleic acid (LA) which are precursors to long-chain (n-3) fatty acids such as eicosapentaenoic acid (EPA) and 21 docosahexaenoic acid (DHA),

respectively (C. Harper, Edwards, DeFilipis, & Jacobson, 2007; Tzang et al., 2009). Both ALA and LA are considered to be essential fatty acids and studies have shown that ALA can protect against cardiovascular disease and inflammation (Bloedon & Szapary, 2004).

It is well known that lipid autoxidation of oils mostly depends on the storage temperature and storage period (Aidos, Lourenco, Van der Padt, Luten, & Boom, 2002; Tan, Man, Selamat, & Yusoff, 2001). H. M. Chen and S. P. Meyers (1982) have reported that the stability of astaxanthin is affected by heat, duration of exposure to oxygen, and the intensity of exposure to light. Therefore, it is important to consider lipid oxidation and degradation rates of flaxseed oil containing astaxanthin, while simultaneously protecting the function and biological activity of the astaxanthin.

The utilization of a novel extraction method to eliminate or reduce the previously mentioned factors that negatively impact the function and stability of lipids and bioactive compounds, like astaxanthin, is ideal for potentially producing a better product. Ultrasound assisted extraction (UAE) technologies are of interest for enhancing the extraction of components from plant and animal tissues, although there is limited information on continuous UAE. Previously published works on UAE collectively represent several important performance increases; reduction in extraction time, increased extraction efficiency, and improved extraction with a reduced extraction temperature (Albu, Joyce, Paniwnyk, Lorimer, & Mason, 2004; Balachandran, Kentish, Mawson, & Ashokkumar, 2006; Riera et al., 2010; Xia, Shi, & Wan, 2006). The objectives of this study were to extract astaxanthin from crustacean (i.e., crawfish and shrimp) processing byproducts using flaxseed oil and to study the oxidation rates and the astaxanthin degradation rates in the flaxseed oil. During the course of this study shrimp processing byproducts were investigated as a source of astaxanthin. However, since the amount

of astaxanthin recovered from shrimp processing byproducts was considerably less than that from crawfish processing byproducts, it was decided to use crawfish processing byproducts for subsequent studies.

2.2 Materials and Methods

Flaxseed oil was purchased from Whole Foods Market (Baton Rouge, Louisiana). DPPH (2,2 diphenyl-1-picryl-hydrazyl-hydrate) and potassium iodide were purchased from Sigma Aldrich (USA), and ascorbic acid was purchased from HiMedia Labs (Mumbai, India. All other chemicals were obtained commercially and were analytical-grade.

2.2.1 Sample collection

The term “crustacean” used here refers crawfish and shrimp, unless specifically stated otherwise. Fresh head-on individually quick frozen (IQF) shrimp (Figures 2.1 and 2.2) were obtained from a local shrimp processor and transported frozen (Figures 2.3) to the laboratory then stored in a freezer until peeled for further analysis and extraction. For analysis, the shrimp were peeled to generate shrimp processing byproducts (SB), containing cephalothorax, carapace, exoskeleton of the abdomen (including pleopods), and viscera. Fresh farmed crawfish, Figure 2.4, were used to obtain crawfish processing byproducts (CB), consisting of the entire cephalothorax, carapace, exoskeleton of the abdomen, and viscera after parboiling (by producer) and removal of the tail meat, Figure 2.5, were obtained from a local Louisiana crawfish producer and processor, and transported to the laboratory frozen on ice. Prior to transportation, CB were sealed in freezer bags (Figure 2.6) at the processing facility, with minimum headspace, and once back at the laboratory placed in a freezer at -20 °C until further analysis and astaxanthin extraction. Prior to ultrasound assisted extraction, the frozen CB (-20 °C) were frozen again at -60 °C, then freeze dried, comminuted using a Magic Bullet® (Capbran Holdings, LLC., Los

Angeles, CA), sieved and stored in the freezer at -20 °C until future analysis.



Figure 2.1. Vats GOM shrimp at processing facility



Figure 2.2. Shrimp being emptied into a container for removal of debris before peeling process.



Figure 2.3. Thawing of IQF shrimp



Figure 2.4. Crawfish farm Crowley, LA.



Figure 2.5. Peeling and sizing crawfish tails.



Figure 2.6. Frozen crawfish byproducts (peelings).

2.2.2 Determining proximate, moisture and total extractable astaxanthin of crustacean processing byproducts

2.2.2.1 Proximate determinations

Three batches of thawed CB or SB were analyzed to determine ash, protein, and fat contents. Two g samples of CB or SB were sent for protein analysis to the SOIL sciences lab, where they were analyzed and ashed in a muffle furnace at 550 °C for 4 h according to AOAC method 942.05 (AOAC 1995). The protein was calculated by multiplying total nitrogen content by 6.25. Fat content was determined using a Soxhlet extraction method according to AOAC method 920.39 (AOAC 1995).

2.2.2.1 Moisture

Moisture content of thawed SB, CB, and freeze-dried CB before and after extraction procedures was determined using a SMART System 5 microwave moisture analyzer (CEM Corporation, Matthews, NC), using a rapid microwave drying method specified in AOAC International procedures for moisture analysis of meat and poultry (AOAC Method 985.14) with minor modifications, i.e. adjusting time to evaporate moisture from sample to prevent burning the sample. The weight loss was determined by electronic balance readings before and after drying.

2.2.2.3 Total extractable astaxanthin in crawfish and shrimp byproducts

The term “astaxanthin” used here refers astaxanthin and all its esters, which have been identified as the main carotenoids in the exoskeleton of heat-processed crustacean. Three batches of comminuted crawfish processing byproducts, thawed comminuted shrimp processing byproducts, freeze dried comminuted crawfish processing byproducts and freeze dried comminuted shrimp processing byproducts were used for the solvent extraction of astaxanthin using a method described by Armenta-López, Guerrero, and Huerta (2002) and H.-M. Chen and

S. P. Meyers (1982) with slight modification. The crustacean processing byproducts were placed in petroleum ether-acetone-water (15:75:10 v/v/v) overnight then filtered. The filtrate was washed with petroleum ether until colorless, and the volume of petroleum ether used was recorded. The absorbance for petroleum ether layer containing astaxanthin was measured at 468 nm (Hong Kyoon No, 1987) using a GENESYS 20 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Rochester, NY, USA). The total extractable astaxanthin in 100 g of crustacean processing byproducts was calculated using equation 2.1 of Kelley and Harmon (1972) and Pu (2010).

$$\text{Astaxanthin yield (mg/100 g byproducts)} = \frac{A_{468} \times V \times 10^4}{W \times E_{\frac{\%}{1\text{cm}}}} \quad (2.1)$$

where A = absorbance at maximum wavelength (468 nm) in petroleum ether; V is volume of petroleum ether (mL); W is weight of crustacean processing byproducts (g); $E_{\frac{\%}{1\text{cm}}}$ is extinction coefficient = 2400 in petroleum ether (H. M. Chen & S. P. Meyers, 1982).

2.2.3 Extraction of astaxanthin from crustacean with flaxseed oil

The effects of pretreatment (freeze-drying, grinding) on the extraction of astaxanthin was initially investigated for verification purposes (data not shown). Two treatment methods are used: conventional, frozen (-20 °C) followed by thawing; and frozen (-20 °C) then frozen (-60 °C) followed by subsequent freeze drying. All freeze dried samples were comminuted in a Magic Bullet® (Capbran Holdings, LLC., Los Angeles, CA) for one minute, then sieved through a 40-mesh screen. Retained shells (unpassed through sieve) were reground with additional shells and subsequently sieved through a 40-mesh screen, followed by sieving through a 30-mesh sieve. Shells passing through the 40-mesh but not the 30-mesh were used.

2.2.3.1 Conventional extraction

For conventional extraction, frozen crawfish processing byproducts were thawed or frozen at -60 °C then freeze dried and used for extraction frozen shrimp were thawed and peeled then the shells were used immediately in the extraction process. Previously. Otherwise CB or SB were kept frozen for later use in extraction of astaxanthin.

Flaxseed oil (FO) containing crawfish astaxanthin (FOCAX) or shrimp astaxanthin (FOSAX) was prepared using a method of N. M. Sachindra et al. (2005) with slight modifications as described in Figure 2.7. Crawfish or shrimp processing byproducts (100 g) were comminuted in a food blender (Magic Bullet®, Capbran Holdings, LLC., Los Angeles, CA), Figure 2.8, for 5 min, then combined with an equal amount of flaxseed oil (100 g) in a 500 mL amber Pyrex® bottle. After simple mechanical extraction (heating of comminuted crustacean processing byproducts/oil mixture 1:1, w/w with constant stirring) for 60 minutes at 60 °C, aliquots (Figure 2.9) of the mixtures were centrifuged at 10,000 x g using a centrifuge (Sorvall RC28S, DuPont, Willmington, DE, USA) for 15 min at 4 °C. This was performed to separate FOCAX or FOSAX and water phase from the solid phase. The top layer lipid fraction was separated manually with a separatory funnel (Figure 2.10) and stored at -20 °C in a sealed container that limits the transmittance of light at -20 °C for later analysis.

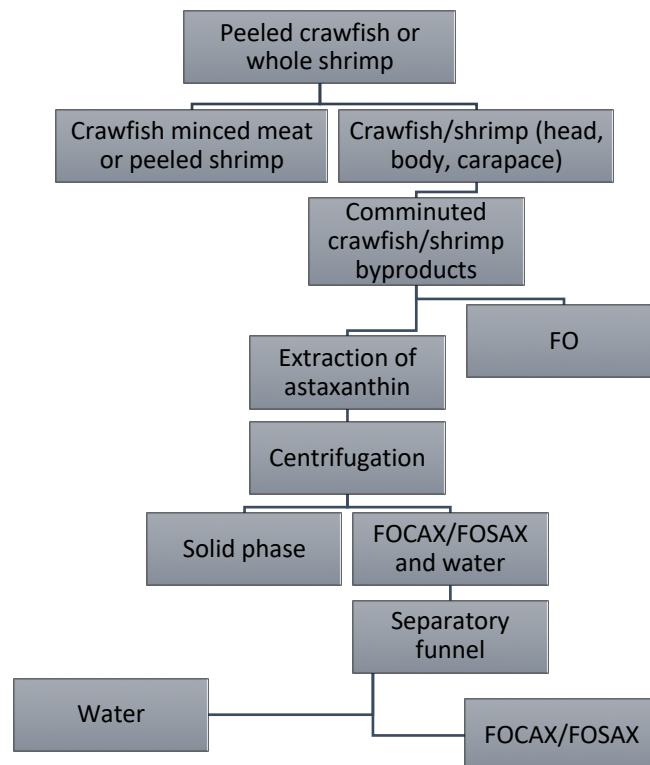


Figure 2.7. Conventional extraction of astaxanthin from crustacean processing byproducts using flaxseed oil. FOCAX = Flaxseed oil containing crawfish astaxanthin. FOSAX = Flaxseed oil containing shrimp astaxanthin.



Figure 2.8. Shrimp processing byproducts.



Figure 2.9. Centrifuge bottle containing flaxseed oil with astaxanthin



Figure 2.10. Phase separation using a separatory funnel



Figure 2.11. Freeze dried crawfish processing byproducts.

2.2.3.2 Novel extraction: combined ethanol, flaxseed oil, and ultrasound assisted extraction of astaxanthin from crawfish processing byproducts

The extraction of AX from crawfish processing byproducts followed a similar procedure as that described in section 2.2.3.1. for crawfish with slight modifications. The crawfish processing byproducts were collected from Bocage Crawfish, LLC (Figure 2.6). These CB were frozen and placed on ice then were transported to the laboratory. The frozen CB was frozen at -60 °C before being freeze-dried, comminuted and sieved (40 mesh). The freeze-dried CB particles were stored in a freezer at -20° C in a 500 mL amber Pyrex bottle. A continuous ultrasonication system was used to provide sonication treatments for releasing AX from the crustacean processing byproducts. The system consisted of an ultrasonic processor (Model CPX 500, Cole Palmer Instruments, Vernon Hills, IL, USA) with an ultrasonic probe of 13 mm diameter and an ultrasonic converter for producing sonic waves at a frequency of 20 kHz (Figure 2.10).

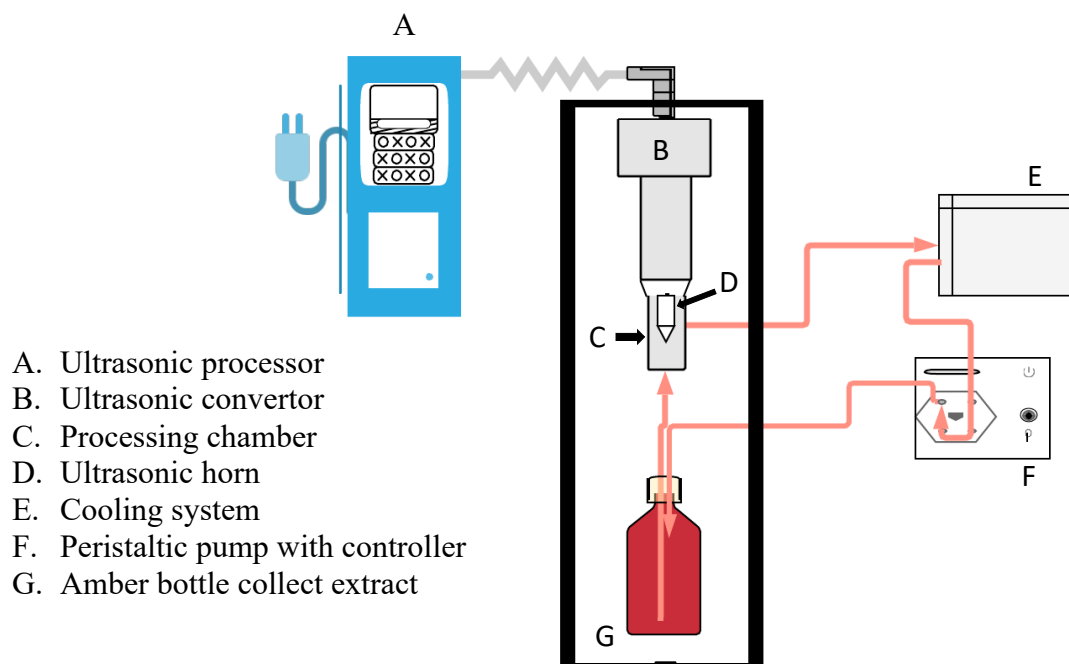


Figure 2.12. Ultrasound assisted extraction system used to extract astaxanthin.

The continuous system was established using a double wall stainless steel cylindrical processing chamber, also called a continuous flow cell (Figure 2.10) which was screwed onto the threaded portion of the $\frac{1}{2}$ inch Ti alloy (Ti-6Al-4V) ultrasonic horn. Freeze-dried comminuted CB (25 g) was placed in the flow cell for AX extraction. The mixture of flaxseed oil with 200 mL of ethanol (1:8), was pumped at the rate of 24 mL/min using a peristaltic pump (Master Flex L/S, Cole Palmer, Vernon Hills, IL, USA) through the collection bottle to the continuous flow cell was CB were sonicated at 40% amplitude by the ultrasonic processor for 20 minutes. Ethanol was selected as a continuous co-solvent for extraction due to its GRAS status (Montanari, Fantozzi, Snyder, & King, 1999). A control sample was prepared by passing only flaxseed oil or only ethanol through the flow cell containing CB without sonication at a rate of 24 mL/min. Prior to the experiment the continuous flow cell was sanitized using 200 ppm solution of sodium hypochlorite (Sigma-Aldrich co., St Louis, MO, USA), followed by rinsing with distilled water to remove residual sanitizer. The temperature of all the samples was maintained at $<25^{\circ}\text{C}$ during sonication using a simple chilling method with dry ice. After

extraction, the crude extracts were vacuum filtered with BUCHI/Whatmann paper #1, 3 times in a dark room, to remove solid particles. The FO containing AX from ethanol and UAE (FOCAXUAE) was concentrated by rotary evaporation to remove residual ethanol and stored in a dark space for immediate analysis. If analyses were to be performed at a later time, samples F were stored in Eppendorf tubes that restrict the passage of light and stored in the freezer at -20 °C until analysis.

2.2.4 Density, specific gravity, water activity, and moisture content of FO, FOCAX, FOSAX and flaxseed oil containing crawfish astaxanthin from ethanol and ultrasound assisted extraction

The density (ρ) of the FO, FOCAX, FOSAX, and FOCAXUAE was determined in triplicate using a 10 mL glass-graduated cylinder at 25 °C. The sample was filled to 10 mL, the mass (g) to volume (mL) ratio determined, and density values ($\rho = m/V$) were reported as grams per milliliter. Specific gravity of the flaxseed oil, FOCAX, FOSAX, and FOCAXUAE samples was determined in triplicate using a 25 mL glass-measuring cylinder. The net weight of the oil sample (g) was divided by the net weight of the same volume of water (g) at 25 °C to obtain the specific gravity. A calibrated water activity (a_w) meter (AquaLab Pawkit, Decagon Devices Inc., Pullman, WA) was used to measure the water activity of flaxseed oil, FOCAX, FOSAX and FOCAXUAE at 25°C. The moisture content was measured according to the Karl Fischer titration method, AOCS Official Method Ca 2e-84, using a Mitsubishi Karl Fischer Moisturemeter (Mitsubishi Chemical Analytech Co., Ltd., Japan). The sample amount size was 0.3 – 0.35 g for FO and 0.25 – 0.36 g for FOCAX, FOSAX, FOCAXUAE.

2.2.5 Measurement of astaxanthin in FOCAX, FOSAX and FOCAXUAE samples.

2.2.5.1 Conventionally extracted astaxanthin concentration

The amount of astaxanthin in the FOCAX and FOSAX was determined spectrophotometrically at 500 nm wavelength as described by Chen and Meyers (1984). The astaxanthin extracted from the processing waste was quantified using Equation 2.2. of Kelley and Harmon (1972) with slight modifications, and reported as mg astaxanthin/100 g flaxseed oil (FO):

$$\text{Astaxanthin (mg/100g FOAX)} = \frac{A_{500} D \times 10^3}{d \times S \times E_{1cm}^{\%}} \quad (2.2)$$

where A is absorbance at 500 nm; D is dilution factor, d is the cell width (1 cm); S = 0.92, specific gravity of flaxseed oil; $E_{1cm}^{\%}$ is extinction coefficient = 1827 in flaxseed oil (Pu et al., 2010); FOAX is flaxseed oil containing crustacean astaxanthin.

2.2.5.2 Combined ethanol, flaxseed oil ultrasound assisted extracted astaxanthin concentration

Astaxanthin concentration (AXC) in the #UAE oil samples was determined according to the method of Saito and Regier (1971) with a slight modification. Oil (30 mg) was mixed with 10 ml of petroleum ether and the mixture was allowed to stand for 30 min. The absorbance of oil, appropriately diluted, was measured at 468 nm using a GENESYS 20 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Rochester, NY, USA). The concentration (C) of astaxanthin in the sample was calculated using Equation 2.3:

$$\text{Astaxanthin } (\mu\text{g/g flaxseed oil}) = \frac{A_{468} \times \text{volume of solution} \times \text{dilution factor}}{0.2 \times \text{weight of sample used (g)}} \quad (2.3)$$

where 0.2 is the A_{468} of 1 $\mu\text{g/ml}$ standard astaxanthin, 10 is the volume of solution in milliliters, and 1 is the dilution factor of solution.

2.2.6 Measurement of color for CB, SB, FO, FOCAX, FOSAX, and UAE oils.

The color of CB, SB, FO, FOCAX, FOSAX, and UAE oils were measured in triplicate using a HunterLab LabScan XE benchtop spectrophotometer (Hunter Associates Laboratory, INC. Resbon, VA) and was reported in CIELAB color scales (L^* , a^* and b^* values). The instrument was standardized using the calibrated black and white standards that come with the instrument. Three grams of flaxseed oil or flaxseed oil with astaxanthin were placed in 1.62 x 1.62 x 0.312 in. polystyrene weighing dish and weighed. Five polystyrene weighing dishes were stacked together to prevent disturbances for the black base. Chroma, hue angle, and color difference (ΔE) values were calculated using Equations (2.4), (2.5) and (2.6), respectively.

$$\text{Chroma} = [a^{*2} + b^{*2}]^{1/2} \quad (2.4)$$

$$\text{Hue angle} = \tan^{-1} (b^*/a^*) \quad (2.5)$$

$$\Delta E_{ab}^* = \sqrt{(L_2^* - L_1^*)^2 + (a_2^* - a_1^*)^2 + (b_2^* - b_1^*)^2} \quad (2.6)$$

(Pagliarini, Vernile, & Peri, 1990)

Where L^* , a^* , and b^* are the degree of lightness to darkness, redness to greenness, and yellowness to blueness, respectively. Negative Hunter a^* values and negative Hunter b^* are converted to positive values for analysis by adding 180° to the arc tangent after conversion to degrees or 360° to the arc tangent after conversion to degrees, respectively, for calculating the proper hue based on the appropriate quadrant (McLellan, Lind, & Kime, 1995).

2.2.7 Measurement of Lipid Oxidation.

2.2.7.1 Peroxide value

Measurement of peroxide values (POV), lipid hydroperoxides, in FO, FOCAX, FOSAX and FOCAXUAE were determined by two titrimetric methods. For FO, FOCAX, and FOSAX samples, the POV were determined as described by Shantha and Decker (1994) using the

International Dairy Federation (IDF) standard method 74A:1991 with slight modifications. POV measures the transient products of oxidation when peroxides form and breakdown, like hydroperoxides formed during the initial stages of lipid oxidation, with high values translating to the instability of an oil during storage. To prepare the iron (II) chloride solution, 0.4 g barium chloride dihydrate was dissolved in 50 mL water. The solution was added slowly while stirring to an iron (II) sulfate solution (0.5 g $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ dissolved in 50 mL water). Then two milliliters of 10N hydrochloric acid was added and the barium sulfate precipitate was filtered off to give a clear iron (II) solution, which was stored in the dark in brown bottles. Thirty grams of ammonium thiocyanate was dissolved in distilled water in an Erlenmeyer flask and brought to volume (100 mL). Peroxide values were calculated as follows:

$$\text{Peroxide value} = \frac{(A_s - A_b) \times m}{55.84 \times m_0 \times 2} \quad (2.7)$$

Where A_s = absorbance of the sample at 500 nm; A_b = absorbance of the blank containing all the reagents except the sample at 500 nm; m = 41.52 (Shantha & Decker, 1994).

The second titrimetric method for determining peroxide value is ideal for small-scale samples. For the UAE samples, the POV was determined in triplicate in accordance with a method of Crowe and White (2001) for small oil samples with slight modifications. An acetic acid-chloroform solution (3:2, v/v) was prepared. A saturated potassium iodide (KI) solution was also prepared by dissolving excess KI in previously boiled distilled water (about 25.0-gram KI in 15.0 mL water). The solution was stored in the dark when not in use. A 0.001 N sodium thiosulfate ($\text{Na}_2\text{S}_2\text{O}_3$) solution was prepared by diluting a 0.1 N $\text{Na}_2\text{S}_2\text{O}_3$ stock (4.962 g $\text{Na}_2\text{S}_2\text{O}_3$ dissolved in distilled water, and brought to 200 mL) 100-fold. Then 50 μL iron (II) solution was added and mixed for 3-4 s. After 5 min incubation at room temperature, the absorbance of the sample (FOCAXUAE) was measured at 500 nm against a blank that contained all reagents but

the sample by using the spectrophotometer. This was conducted under dim light and completed within 10 minutes. The POV, expressed as millimoles of peroxide ($mmol O_2^{2-}$) per kilogram of sample, was calculated using the following formula:

$$\text{Peroxide value} = \frac{(S-B) \times N \times 1000}{\text{mass of sample, g}} \quad (2.8)$$

2.2.7.2 Thiobarbituric acid-reactive substances (TBARS) analysis of FO, FOCAX, FOSAX, and UAE extractions

TBARS content of FO, FOCAX, FOSAX, and all FOCAXUAE (50UAE0, 25UAE, and 10UAE) sample were measured using methods described by Mei, McClements, Wu, and Decker (1998) and Lemon (1975) with modifications. TBA (thiobarbituric acid) solutions were prepared by mixing 15 g of trichloroacetic acid, 0.375 g of TBA reagent, 1.76 mL of 12 N HCl, and 82.9 mL of distilled H₂O. Every 100 mL of TBA solution was mixed with 3 mL of 2% butylated hydroxytoluene in ethanol. A 0.1 g sample of oil was added to 0.4 mL of water then combined with 2.5 mL of TBA solution in a 15 mL capped glass tube vortexed and placed in boiling water (95-100 °C) for 15 min. After cooling to room temperature, the mixture was centrifuged at 1163 × g for 25 min using a benchtop centrifuge (Compact II Centrifuge, Becton-Dickinson, Rutherford, New Jersey, USA) and absorbance was measured at 532 nm. TBA values were calculated from a standard curve prepared using TEP (1,1,3,3-tetraethoxypropane) standard and expressed as mg malondialdehyde (MDA) kg⁻¹oil.

2.2.8 Studies on oxidative stability of FO, FOCAX, FOSAX, and FOCAXUAE

A modified method of Ragnarsson and Labuza (1977) was employed to evaluate the ability of astaxanthin as an antioxidant to protect flaxseed oil from detrimental effects of elevated temperature over time. One hundred twenty-five mL amber Erlenmeyer flasks containing 50±2 g of oil samples each was used. The flasks were placed in a water bath at 65 °C to model

accelerated shelf life (ASL), while other amber flasks containing oil samples, were not exposed to heat, and placed at room temperature 25 ± 0.2 °C. An oil sample was taken from the amber flasks using a pipette every hour from 0 to 4 hours for POV. A plot of POV versus time was constructed for 65 °C. The resultant straight line yielded the of oxidation rate ($mEq O_2^{2-} \cdot kg \text{ oil}^{-1} \cdot h^{-1}$) at 65 °C.

2.2.9 Free fatty acids of FO, FOCAX, FOSAX, and FOCAXUAE

Free fatty acid (FFA) content was determined in triplicate for each sample according to a titrimetric method of Ke, Woyewoda, Regier, and Ackman (1976) with slight modification. One g samples of oil before and after extracting AX was placed using a dropper and weighed (± 0.005 g) on an analytical benchtop scale in an Erlenmeyer flask and 75 mL of ternary solvents (Chloroform, Methanol, Isopropyl Alcohol; 2:1:2) were added, followed by 4 drops of meta-cresol purple (MCP) indicator solution. As the sample was being mixed on a stir plate, the FFAs were titrated to the purple endpoint with 0.05 N NaOH using a burette. The blank was ternary solvents and MCP. The percentage of FFA was expressed as oleic acid equivalents using the equation described by Ke et al. (1976).

$$FFA \text{ as } \% \text{ oleic} = \frac{(T-B) \times 1.41}{S} \quad (2.9)$$

Where T and B are the volume (ml) of 0.05 N NaOH used to titrate the sample and the blank, respectively, and S is the weight (g) of sample.

2.2.10 Vitamin C equivalent antioxidant capacity (VCEAC) of FO, FOCAX, FOSAX, and FOCAXUAE samples in scavenging DPPH (2,2 diphenyl-1-picryl-hydrazyl-hydrate) radical, a spectrophotometric assay

The radical-scavenging activity of ethanolic ultrasound-assisted extracted AX in FO was assayed following a method described by Rao, Sarada, and Ravishankar (2007) with some modifications. A stable free radical, DPPH (2, 2-diphenyl-1-picryl-hydrazyl), was used to

evaluate the free radical scavenging capability of FO, FOCAX, FOSAX, and FOCAXUAE samples. DPPH reagent (2.36 mg) was dissolved in 100 mL of ethanol in an Erlenmeyer flask, in the dark, with 10 min sonication to ensure complete solubilization in the dark. The ethanolic DPPH solution (60 μ M) was kept in the dark. One mL of the DPPH solution added to 3 mL of FO, FOCAX, FOSAX, and FOCAXUAE extracts. The percent of DPPH radical-scavenging activity, or inhibition, was calculated as:

$$DPPH \text{ scavenging activity (\%)} = \frac{A_{control} - (A_{sample} - A_{sample \text{ blank}})}{A_{control}} \times 100 \quad (2.10)$$

where $A_{control}$, A_{sample} , and $A_{sample \text{ blank}}$ are the absorbance of the DPPH solution (1.0 mL: 60 μ M) plus ethanol, astaxanthin solution with DPPH (different concentration) and astaxanthin solution containing ethanol without DPPH, respectively. The absorbance measurements were taken at 517 nm.

2.2.11 Preparation of standard curve for the absorbance of DPPH against

A Vitamin C (ascorbic acid) calibration curve that relates the concentration of vitamin C and the amount of absorbance reduction caused by vitamin C were obtained using the DPPH assay. In order to express DPPH radical scavenging results as mg Vitamin C L⁻¹ oil a calibration curve was prepared using 0.1 mL ethanolic solutions of ascorbic acid in the range 0–113.5 mM or 20 mg. The linearity was found up to 113.5 mM concentration of ascorbic acid and a line was calculated (Figure 2.11). The absorbance at 0-30 minutes of incubation with DPPH radical (2.4 mL) was recorded. The absorbance reduction was measured at 517 nm for the astaxanthin extracts (FO, FOSAX, FOCAX, and FOCAXUAE against the various concentrations DPPH radical to then determine the VCEAC equivalent of each extract.

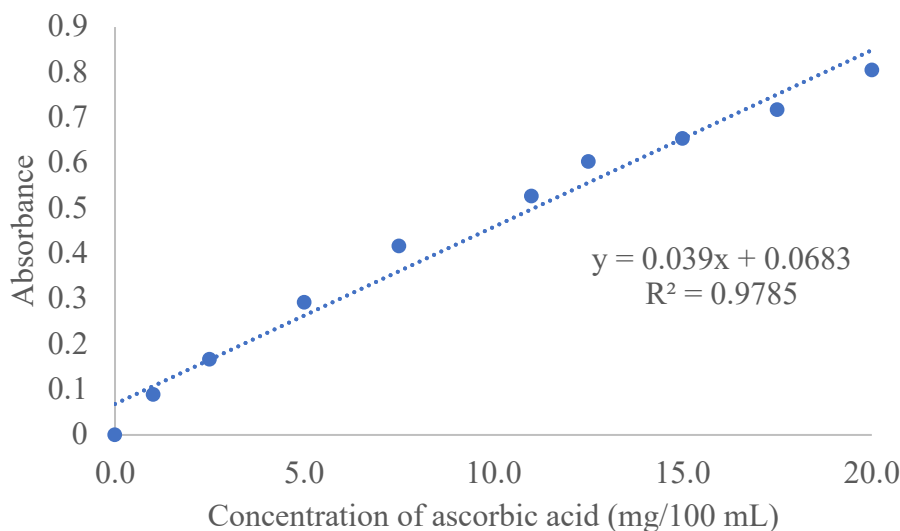


Figure 2.13. Calibration curve using ascorbic acid.

Calibration curves of absorbance reduction of DPPH radicals versus vitamin C concentration were generated. The decreasing absorbances at 517 nm for DPPH radical scavenging activity by FO, FOCAX, FOSAX, and FOCAXUAE were measured at various concentrations. The results were converted into VCEAC (Vitamin C equivalent antioxidant capacity) using the vitamin C calibration curve. The scavenging activities of tested compounds against DPPH radicals were calculated as VCEAC in mg/100 ml.

Ascorbic acid was dissolved in ethanol to make 0 – 20 mg 100 ml⁻¹ concentrations and plotted vs. absorbance at 517 nm. Samples were prepared in triplicate.

2.2.12 Statistical analysis

Mean values and standard deviations of triplicate reproducible analyses are reported. The statistical significance of observed differences among treatment means was evaluated by analysis of variance (ANOVA) was carried out to determine the difference among treatment means with SAS software Version 9.4 (SAS Institute Inc., Cary, NC) using a *post hoc* Tukey's studentized range test (SAS, 2013) at a significance level of 0.05.

2.3 Results and Discussion

2.3.1 Proximate compositions and total extractable astaxanthin of crustacean processing byproducts.

Moisture content (wet weight basis, ww) of crawfish and shrimp and crawfish processing byproducts was $53.69 \pm 0.1\%$ and $74.82 \pm 0.02\%$, respectively. The moisture content (dry byproducts, db) of crawfish and shrimp processing byproducts was $2.92 \pm 0.01\%$ and 3.19 ± 0.03 , respectively. The moisture content (ww) for thawed shrimp processing byproducts is higher, but for thawed crawfish processing byproducts is similar to that reported in previous works by Pu (2010), 62.82% and 54.68%, respectively. Furthermore, the moisture content of the produced solid waste after conventional extraction of astaxanthin from crawfish and shrimp, was 57.69% and 60.04 %, respectively, while after UAE extraction of AX from crawfish processing byproducts their moisture content was 46.31 %. The values from conventionally extracted crustacean byproducts were higher than those reported by Pu (2010) for shrimp, 62.82 %, and crawfish, 54.68 %.

Ash, fat, and protein contents (db) of crawfish processing byproducts before extraction were, 40.76 ± 0.05 %, 6.6 ± 0.06 %, and 37.93 ± 0.2 %, respectively (Table 2.1). The ash content was within 5 % of values reported by authors in other works, 42.97% (Pu, 2010) and 35.7 % (Meyers & Bligh, 1981). However, the fat content was very close to that other authors reported, 6.7 % (Meyers & Bligh, 1981). The total ash, fat, and protein contents for the shrimp processing byproducts accounts for 88.29 %, resulting in the remaining 14.71 % possibly being chitin.

Ash, fat, and protein contents (db) of shrimp processing byproducts before extraction were 18.14 ± 0.68 %, 7.93 ± 0.33 %, and 57.1 ± 0.57 %, respectively (Table 2.1). Previously reported ash, fat, and protein content values were, 17.41%, 7.22%, 60.2%, respectively, and 22.2%, 22.7%, and 51.3%, respectively (Lee and others, 1999). The proximate composition of

shrimp processing byproducts varies, depending on their age, diets, species or native habitats, which depend on the season (N. M. Sachindra et al., 2007; Yanar, Çelik, & Yanar, 2004). The total ash, fat, and protein contents for the shrimp processing byproducts accounts for 83.17 %, resulting in the remaining 16.83 % potentially being chitin.

Astaxanthin being lipophilic, is solubilized in lipids. Therefore, the quantification of astaxanthin can be approached from a comparable assessment to extracting lipids with solvents. Likewise, extraction was performed to measure the total amount of extractable astaxanthin in the crawfish (thawed) processing byproducts, shrimp (thawed) processing byproducts, crawfish (freeze dried) processing byproducts, and shrimp (freeze dried) processing byproducts. The result was 8.53 ± 0.15 mg 100 g⁻¹ waste, 13.83 ± 0.31 mg 100 g⁻¹ waste, 10.21 ± 0.04 mg 100 g⁻¹, and 18.68 ± 0.04 mg 100 g⁻¹ freeze-dried waste, respectively. The total AX content for wet but not freeze-dried crawfish was slightly lower than that extracted using the same solvents, petroleum ether-acetone-water mixture, to extract astaxanthin from crawfish waste, 15.3 mg of astaxanthin 100 g⁻¹ of crawfish processing byproducts (Meyers & Bligh, 1981). After comparing the total extractable astaxanthin data, it was determined that crawfish processing byproducts would be used for all subsequent studies.

2.3.2 Physicochemical characteristics and astaxanthin content of FO, FOCAX, FOSAX, and FOCAXUAE

The FO, FOCAX, FOSAX, and FOCAXUAE densities (g/mL) were 0.91 ± 0.00 , 0.92 ± 0.07 , 0.92 ± 0.02 and 0.91 ± 0.03 , respectively (see Table 2.2), which was a little higher than values reported by Sathivel (2005) for red salmon oil (0.9 g/mL) and pink salmon oil (0.81 g/mL). Specific gravities of the FO, FOCAX, FOSAX, and FOCAXUAE were 0.92 ± 0.00 at room temperature. The moisture contents of the FO, FOCAX, and FOSAX were $0.15 \pm 0.00\%$, $0.17 \pm 0.03\%$, $0.17 \pm 0.05\%$, while in FOCAXUAE the moisture content was $0.15 \pm 0.03\%$. The

water activity of FO (0.51 ± 0.0), FOCAX (0.52 ± 0.0), FOSAX (0.52 ± 0.0), and UAEAX (0.51 ± 0.7) was slightly lower than other values reported by Sathivel (2005) for red salmon oil (0.57) and pink salmon oil (0.53). Moisture in oil increases the FFA content of the oil by lipid hydrolysis reactions (Bhattacharya and others 2008). Water contributes to the hydrolysis of oil during various handling and processing steps, which generate free fatty acids and glycerol products that decrease the oxidative stability of oils (Bhattacharya, Sajilata, Tiwari, & Singhal, 2008; Choo, Birch, & Dufour, 2007b). FFA content is an intrinsic factor that impacts oxidative stability and shelf-life of oils. Therefore, it is most desired to have an oil with a low a_w value.

For conventional extraction using FO, the astaxanthin concentration (AXC) in FOCAX (1:1, CB: oil), FOSAX (1:1, SB: oil) and the FOCAXUAE (1:1:8, 25g CB: 25g oil: 200g ethanol) per gram of flaxseed oil were, 0.054 mg g^{-1} , 0.092 mg g^{-1} , and 1.9 mg g^{-1} , respectively (Table 2.3), which are similar to values previously reported in the literature for shrimp processing byproducts, 0.049 mg g^{-1} dry waste by De Holanda and Netto (2006) and (De Holanda & Netto, 2006; Handayani, Indraswati, & Ismadji, 2008) and crawfish processing byproducts (Meyers & Bligh, 1981). Conversely, the values were greater than those reported by Pu (2010) for crawfish and shrimp, 0.0884 mg g^{-1} wet CB and 0.0647 mg g^{-1} wet SB, respectively. The conventionally extraction technique used here was similar to that of Pu (2010) with slight modification. Key differences to highlight include pre-treatments applied like comminuting and freeze-drying. In a previous investigation, De Holanda and Netto (2006) used an enzymatic method to extract astaxanthin from shrimp (*Litopenaeus setiferus*) processing byproducts using soy oil and extracted $4.90 \text{ mg astaxanthin } 100 \text{ g}^{-1}$ processing byproducts. The amount of conventionally extracted astaxanthin in both FOCAX and FOSAX was greater than that in other vegetable oils such as sunflower oil, groundnut oil, coconut oil, and rice bran

oil obtained by N. Sachindra and Mahendrakar (2005). Meyers and Bligh (1981) reported a 10-fold increase in astaxanthin concentration with vacuumed dried shrimp processing byproducts (0.100 mg/g) compared to heat dried shrimp processing byproducts (0.010 mg/g). H.-M. Chen and S. P. Meyers (1982) compared the astaxanthin recovery between heat processed CB (0.1615 mg/g) and unprocessed (not heat processed) CB (0.0878 mg/g) using standard organic solvents for extraction. They found that pretreatment with a sonifier and particle size reduction contributed to the increase in recovery. Still, the amount of conventionally extracted astaxanthin from crawfish (FOCAX) and FOCAXUAE in this study was greater than that obtained by Chen and Meyers.

The amount of astaxanthin recovered by UAE ratios were 0.8 mg g⁻¹ lipid, 1.9 mg 1 g⁻¹ lipid, and 0.9 mg g⁻¹ lipid, for ratios CB:FO to ethanol (E) 1:1:4 (A), 1:1:8 (B), and 1:1:20 (C), respectively. While the amount of astaxanthin recovered by conventional extraction were, 0.054 mg g⁻¹ FO (FOSAX), 0.092 mg g⁻¹ FO (FOCAX). Both CFO, 0.1 mg g⁻¹, or NFO, 0.2 mg g⁻¹, alone yielded lower extractions of AX than did the combination of the two within the closed system. Although, the extraction yields were still greater than the conventional method of extraction with regard to the amount flaxseed oil and crustacean byproducts used.

Numerous studies have revealed that the yield of astaxanthin depends upon many factors; such as the extraction methods, the solvents or media used for extraction, and other factors such as particle size of the waste and ratio of waste to oil used for extracting astaxanthin from crustacean processing byproducts (Praveenkumar, Lee, Lee, & Oh, 2015; N. Sachindra et al., 2006). However, for UAE, ethanol (E) was combined with the flaxseed oil to extract astaxanthin, which explains why FOCAXUAE had a higher concentration of astaxanthin than FOCAX and FOSAX. E was chosen as a co-solvent for the ultrasound-assisted extraction of AX, and was

previously shown to be the best solvent for extracting AX (Bi, Tian, Zhou, & Row, 2010). Ethanol, a very polar short chain alcohol lacks FDA regulatory problems, unlike many other solvents. It is the least toxic of the alcohols and is suitable for use in industry and consumer products. Previous studies also give supporting data for greater extraction yields of astaxanthin obtained when organic solvents were used (Ali-Nehari, Kim, Lee, Lee, & Chun, 2012; Reyes, Mendiola, Ibanez, & del Valle, 2014). The differences in extractions of AX could be attributed to the additional reduction in the moisture content of the CB by E. Polar solvents (i.e., ethanol) will help remove the water in the solid matrix making the less polar pigment (i.e., astaxanthin) more available for extraction in non-polar solvents (N. Sachindra et al., 2006).

Table 2.1. Proximate compositions and AX content of CB, SB, freeze-dried CB, and freeze-dried SB.

Treatment	Astaxanthin (mg g-100 byproducts)	% Moisture (wwb)	% Moisture (db)	Ash %	Fat %	Protein %
SSBW	8.53±0.15 ^A	74.82±0.02 ^A		18.14±0.68 ^A	7.93±0.22 ^A	57.1±0.57 ^A
SCBW	13.83±0.31 ^B	53.69±0.01 ^A		40.76±0.05 ^B	6.6±0.06 ^A	37.93±0.2 ^B
SFDSB	10.21±0.04 ^D		3.19±0.02 ^A			
SFDCB	18.68±0.04 ^C		2.92±0.01 ^A			

Note: Values are means ± SD of triplicate determinations. SSBW = Solvent extraction of 100 g thawed shrimp processing byproducts; SCBW = Solvent extraction of 100 g thawed crawfish processing byproducts; SFDSB = Solvent extraction of 100 g freeze dried shrimp processing byproducts SFDCB = Solvent extraction of 100 g freeze dried crawfish processing byproducts.¹

¹ For SFDSB and SFDCB, the ash, fat, and protein data are not shown because the determinates would not differ.

Table 2.2. Physicochemical properties of FO, FOCAX, FOSAX, and FOCAXUAE.

	FO	FOSAX	FOCAX	FOCAXUAE
Density	0.91±0.00 ^A	0.92±0.07 ^A	0.92±0.02 ^A	0.91±0.03 ^A
S.G.	0.92±0.00 ^A	0.92±0.00 ^A	0.92±0.00 ^A	0.92±0.00 ^A
Moisture %	0.15±0.00 ^A	0.17±0.03 ^B	0.17±0.05 ^B	0.15±0.03 ^A

Note: Values are means ± SD triplicate determinations. Values not containing the same letters in the same row are significantly different (P<0.05). FO = Flaxseed oil; FOCAX = Flaxseed oil containing crawfish astaxanthin from conventional extraction; FOSAX = Flaxseed oil containing shrimp astaxanthin from conventional extraction; FOCAXUAE = Flaxseed Oil Containing Crawfish Astaxanthin From Ethanol and Ultrasound Assisted Extraction. S.G. = specific gravity.

Table 2.3. AXC, POV and TBARS of FO, FOSAX, FOCAX, 10UAE, 25UAE and 50UAE with FOAX, NFO and CFO.

Treatment	AXC (mg g ⁻¹ oil)	POV (mmol O ₂ · kg ⁻¹)	TBARS (mg MDA · kg ⁻¹)	FFA
FOSAX*	0.054±0.403 ^E	1.84±0.05 ^A	1.49±0.02 ^F	2.85±0.01 ^D
FOCAX*	0.092±0.912 ^C	1.64±0.09 ^A	1.39±0.07 ^E	2.17±0.03 ^B
UTCB**	2.1±1.34e-4 ^D	1.35±0.83 ^B	2.38±0.01 ^A	2.13±0.06 ^B
UTO (FO)	NA	1.27±0.55 ^B	2.09±0.04 ^A	1.57±0.01 ^C
50UAE**	0.8±1.86e-5 ^B	1.75±1.61 ^C	1.95±0.00 ^C	2.01±0.01 ^A
25UAE**	1.9±2.44e-4 ^A	1.59±0.33 ^A	1.77±0.55 ^B	1.96±0.04 ^A
10UAE**	0.9±3.41e-5 ^B	1.35±0.60 ^B	1.55±0.03 ^D	1.94±0.11 ^A
NFO**	0.2±1.74e-6 ^C	2.49±1.04 ^B	2.09±0.03 ^A	
CFO**	0.1±9.58e-6 ^C	4.21±1.11 ^B	2.11±0.03 ^E	2.48±0.94 ^C

Values are means of triplicate determinations. ^{A-E} Means ± SD containing the same letters in the same column are not significantly different (P<0.05). AXC = Astaxanthin concentration. POV = Peroxide value after the 60-minute conventional extraction time or 20-minute UAE time; TBARS = Thiobarbituric acid reactive substances. FFA = Free fatty acids. FOCAX = Flaxseed oil containing crawfish astaxanthin from conventional extraction; FOSAX = Flaxseed oil containing shrimp astaxanthin from conventional extraction; FO = Flaxseed oil; CB = crawfish processing byproducts; UAE = ultrasound-assisted extraction with ethanol; 50UAE = 50 g CB with 50 g FO, 25UAE = 25 g CB with 25 g FO, 10UAE = 10 g CB with 10 g FO; UTCB = Non-UAE oil with ethanol 25 g CB; UTO = Non-UAE oil with ethanol without CB; NFO = EtOH only with 25 g CB; CFO = Just FO only with 25 g CB; For NFO, 3g of flaxseed oil used for quantitative measurement of AX and oxidative stability. * denotes POV determined by IDF 74A:1991 method. ** denotes POV determined by method of Crowe and White (2001).

2.3.3 Color evaluation of FO, FOCAX, FOSAX, and FOCAXUAE

All color values (L^* , a^* , b^* , chroma, hue angle, ΔE) of CB and SB before and after extraction, as well as FO, FOCAX, FOSAX and FOCAXUAE are shown in Table 2.4. The L^*

value is a measure of the lightness (0-100), while a^* and b^* values are the measurement of redness (more +) and yellowness (more +), respectively. The L^* values of all the oil-based samples are slightly darker (FOCAX = 45.74; FOSAX = 48.57; 10UAE = 31.17; 25UAE = 39.39; 50UAE = 41.87) in color than FO, 49.02 and HFO = 52.32. Conversely, the a^* values of all the oil-based samples were determined to have more redness (FOCAX = 11.34; FOSAX = 8.84; 10UAE = 33.61; 25UAE = 39.76; 50UAE = 30.89) in color than FO, 1.88. The b^* values of all the oil-based samples were determined to have less yellowness (FOCAX = 64.68; FOSAX = 73.08; 10UAE = 60.07; 25UAE = 65.35; 50UAE = 53.98) than FO, 77.34. As it relates to color measurements for the crustacean processing byproducts, the color values of L^* , a^* and b^* are representative of the extraction processes. The SB before extraction had L^* , a^* and b^* values of, 60.71, 0.15 and 5.22, respectively. CB had L^* , a^* and b^* values of, 53.68, 57.86, and 13.88, respectively. Conversely, the SB after extraction L^* , a^* and b^* values were, 58.39, 20.09 and 45.76, respectively, and after extraction, the CB L^* , a^* and b^* values were, 48.47, 5.34, and 14.2, respectively. There was a decrease in redness of CB. This may be attributed to the removal of astaxanthin from solid material. Chroma is a measurement of the vividness of color (higher values indicate a more vivid color). The chroma values for FO, FOCAX, FOSAX, and 25UAE were, 77.35, 73.71, 73.62, and 76.49, respectively (Table 2.3). Hue angle is defined as a color wheel with red-purple at an angle 0° and yellow at 90° (Ergüneş & Tarhan, 2006) The hue angle values for FO, FOCAX, FOSAX, and 25UAE were, 88.61, 81.15, 83.1, and 58.68, respectively (Table 2.3). Of the UAE samples, 25UAE had higher chroma value than 50UAE and 10UAE, indicating that 25UAE had a more vivid color than 50UAE and 10UAE.

The greater the color difference amongst the oil samples correlated with the greater astaxanthin yield. FOCAX and FOSAX (conventional extraction) and, FO. The FOCAXUAE oils were referenced to FO within treatments for determining the color differences.

Table 2.4 – Color in terms of L^* , a^* , b^* , Chroma, Hue, and color difference of FOSAX, FOCAX, HFO and FO.

Sample	L^*	a^*	b^*	C*	h^*	Color difference (ΔE_{ab}^*)
FO	49.03±0.15 ^A	1.88±0.2 ^C	77.34±0.36 ^A	77.35±0.94 ^A	88.61±1.94 ^A	NA
HFO	52.32±0.25 ^B	1.40±1.16 ^B	62.82±0.67 ^B	62.84±0.49 ^A	88.72±0.75 ^A	2.51±0.14 ^A
FOAX	29.78±0.94 ^E	47.07±0.94 ^D	60.00±0.94 ^B	76.26±0.12 ^C	51.89±0.94 ^A	NA
FOSAX	48.57±0.15 ^A	8.85±0.15 ^A	73.08±0.94 ^A	73.62±0.85 ^A	83.1±0.79 ^A	8.18±0.05 ^A
FOCAX	45.74±0.15 ^A	11.34±0.56 ^A	64.68±0.19 ^B	73.71±0.65 ^A	81.15±2.01 ^A	10.98±0.09 ^A
50UAE	41.87±0.94 ^A	30.89±1.39 ^D	53.98±0.07 ^B	62.19±0.92 ^B	60.22±1.63 ^B	37.92±0.71 ^B
25UAE	39.39±0.13 ^D	39.76±1.43 ^D	65.35±1.32 ^B	76.49±1.53 ^A	58.68±1.15 ^B	40.88±0.36 ^B
10UAE	31.17±0.147 ^D	33.61±1.43 ^D	60.07±1.76 ^B	68.83±1.25 ^B	60.77±1.31 ^B	40.29±0.23 ^B
SB	60.71±0.22 ^C	0.15±0.02 ^B	5.22±0.99 ^C	5.22±0.67 ^C	88.35±3.19 ^A	NA
SBA	58.39±1.59 ^C	20.09±1.23 ^C	45.76±1.03 ^C	49.98±0.87 ^D	66.30±0.67 ^A	45.24±0.82 ^B
CB	53.68±1.43 ^B	57.86±2.67 ^E	13.88±0.99 ^D	59.5±1.75 ^C	13.49±0.91 ^C	NA
CBA	48.47±1.55 ^B	5.34±0.81	14.2±1.63 ^D	15.17±0.83 ^E	69.39±0.14 ^B	52.79±0.04 ^B

Values are means of three determinations. ^{A-E} Means within same column bearing different letters are significantly different ($p<0.05$). FOSAX = Flaxseed oil containing astaxanthin heated at 65° C for 1 h; FOCAX = Flaxseed oil containing astaxanthin heated at 65° C for 1 h; FOCAXUAE = Flaxseed oil containing crawfish astaxanthin from ultrasound assisted extraction heated at 65° C for 1 h; HFO = Flaxseed oil heated at 65° C for 1 h; FO = Flaxseed oil; SB = shrimp processing byproducts (wet) before extraction; CB = crawfish processing byproducts (wet) before extraction; SBA = shrimp processing byproducts (wet) after extraction; CBA = crawfish processing byproducts (wet) after extraction; 50UAE = 50 g CB with 50 g FO, 25UAE = 25 g CB with 25 g FO, 10UAE = 10 g CB with 10 g FO L^* = lightness ; a^* = redness; b^* = yellowness; C* = chroma or vividness; h^* = hue angle.

The changes in the lightness (L^*), redness (a^* value) and yellowness (b^* value) for the extraction methods considered are shown in Figures 2.12, 2.13, and 2.14. Lightness does not seem to be affected by extraction conditions; slightly different L^* values were recorded for the oils utilized for UAE and the oils utilized for conventional extraction. The most significant differences are observed in a^* values, which are related to the tonality of color, possibly an increase in AX content of the oil, release of AX from the carotenoprotein matrix, or removal of AX from the shell matrix. 25UAE had higher redness ($a^* = 39.76$) after extraction than did CFO ($a^* = 0.26$). CFO (control, flaxseed oil only with ultrasound assisted extraction using 25 grams of crawfish processing byproducts) was lighter and less yellow ($L^* = 48.45$ and $b^* = 37.15$) in color than 50UAE, 25UAE, and 10UAE ($L^* = 43.31, 39.38,$ and 43.31 and $b^* = 53.98, 65.35,$ 60.07 , respectively). The study demonstrated that the closed ultrasound assisted extraction system combining ethanol with flaxseed oil could be an effective method to efficiently improve the extraction of astaxanthin using only 25 g of flaxseed oil and 25 g crawfish processing byproducts, thus increasing yield.

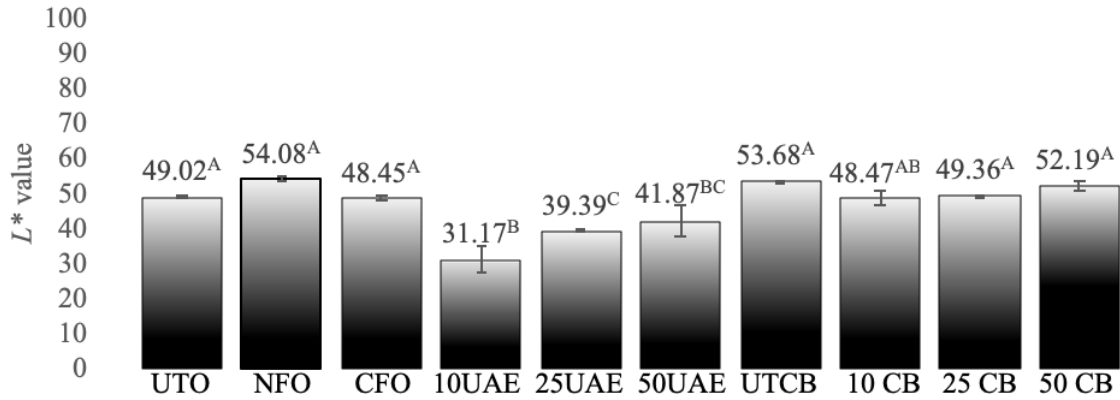


Figure 2.14. L^* value, measurement of lightness to darkness in terms of CIELAB color scale. L^* means \pm SD values of triplicate determinations. ^{A-C} Means \pm SD not containing a letter in common are significantly different ($p=0.05$). UTO = flaxseed oil only pumped through system without CB and no UAE; NFO = ethanol only pumped through system with CB and no UAE; 10UAE = 10 g flaxseed oil containing AX post UAE of 10 g CB; 25UAE = 25 g flaxseed oil containing AX post UAE of 25 g CB; 50UAE = 50 g flaxseed oil containing AX post UAE of 50 g CB; 10 CB = 10 g crawfish processing byproducts (wet) after extraction; 25 CB = 25 g crawfish processing byproducts (wet) after extraction; 50 CB = 50 g crawfish processing byproducts (wet) after extraction.

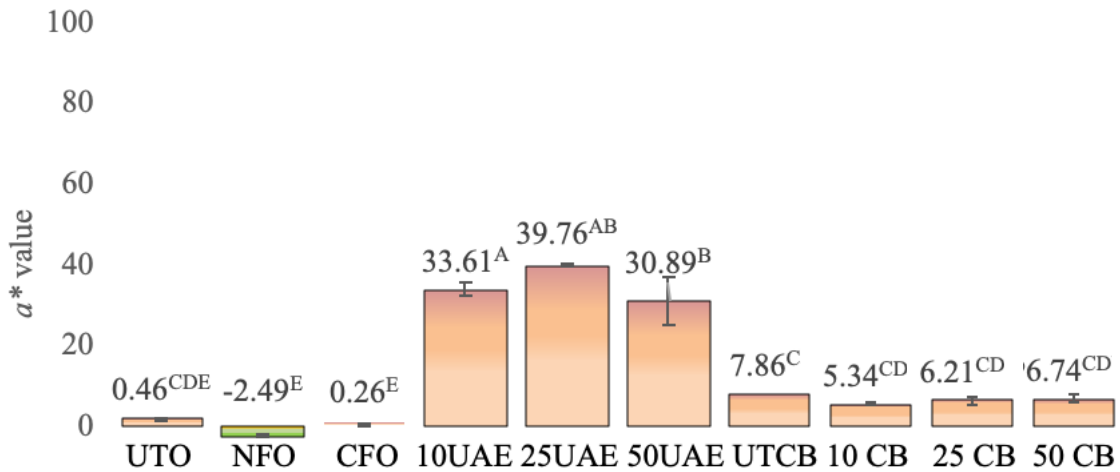


Figure 2.15. a^* value, measurement of red to green in terms of CIELAB color scale. a^* means \pm SD values of triplicate determinations. ^{A-E} Means \pm SD not containing a letter in common are significantly different ($p=0.05$). UTO = flaxseed oil only pumped through system without CB and no UAE; NFO = ethanol only pumped through system with CB and no UAE; CFO = flaxseed oil without ethanol for UAE; 10UAE = 10 g flaxseed oil containing AX post UAE of 10 g CB; 25UAE = 25 g flaxseed oil containing AX post UAE of 25 g CB; 50UAE = 50 g flaxseed oil containing AX post UAE of 50 g CB; 10 CB = 10 g crawfish processing byproducts (wet) after extraction; 25 CB = 25 g crawfish processing byproducts (wet) after extraction; 50 CB = 50 g crawfish processing byproducts (wet) after extraction.

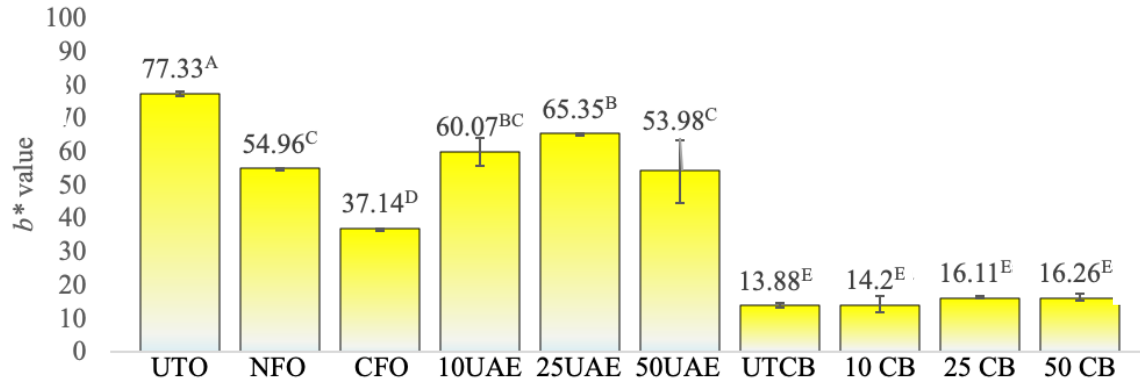


Figure 2.16. b^* value, measurement of yellow to blue in terms of CIELAB color scale. B^* means \pm SD values of triplicate determinations. ^{A-E} Means \pm SD not containing a letter in common are significantly different ($p=0.05$). UTO = flaxseed oil only pumped through system without CB and no UAE; NFO = ethanol only pumped through system with CB and no UAE; CFO = flaxseed oil without ethanol for UAE; 10UAE = 10 g flaxseed oil containing AX post UAE of 10 g CB; 25UAE = 25 g flaxseed oil containing AX post UAE of 25 g CB; 50UAE = 50 g flaxseed oil containing AX post UAE of 50 g CB; 10 CB = 10 g crawfish processing byproducts (wet) after extraction; 25 CB = 25 g crawfish processing byproducts (wet) after extraction; 50 CB = 50 g crawfish processing byproducts (wet) after extraction.

2.3.4. Oxidative stability and quality of FO, FOSAX, FOCAX, and FOCAXUAE.

The quality of the oil samples was evaluated by color, water content, content of free fatty acids (FFA), peroxide value (POV), thiobarbituric acid reactive substances (TBARS), and antioxidant capacities of FO, FOSAX, FOCAX, and FOCAXUAE. Following the extraction procedures, samples of the oils were taken for assessment of oxidative stability as a quality parameter. The peroxide value measures the degree of oxidation in fats and oils and measures the amount of total peroxides as a product of primary oil oxidation (Farhoosh & Moosavi, 2009). POV is a representation of the quantity of active oxygen (in mg) within 1 g of lipid that could oxidize potassium iodide releasing iodine. In the conventional extraction of AX from SB and CB, the POV of FO, FOCAX, and FOSAX were 1.27, 1.64 and 1.84 mmol $O_2 \cdot Kg^{-1}$, respectively. The POV of 50UAE, 25UAE, and 10UAE were 1.75, 1.59, and 1.35 mmol $O_2 \cdot kg^{-1}$ of oil. These values were lower than that of CFO, 4.27 mmol $O_2 \cdot kg \text{ oil}^{-1}$. The POV of FO (UTO), 0.64 mmol $O_2 \cdot kg \text{ oil}^{-1}$, was similar to that reported by Symoniuk, Ratusz, and Krygier

(2016), who measured POV ranging from 0.78 – 1.11 mmol O₂ ·kg oil⁻¹. Initial results for FO, FOCAX, FOSAX, and FOCAXUAE showed that the oils used for extraction developed a small amount of peroxides. Although oils containing AX underwent oxidation during both conventional and novel extraction methods, extraction using the latter method resulted in a lower POV value. Additionally, peroxide values are used as a measurement of rancidity of oils which occur by auto oxidation. Furthermore, since the carotenoid portion may also be degraded during lipid peroxidation processes, the measurement of POV in the oils containing carotenoids may also provide information about color stability (Hornero-Méndez, Pérez-Gálvez, & Mínguez-Mosquera, 2001). Greater exposure to oxygen in conventional extraction processes yielded more than the minimal oxidation created by the low exposure to oxygen in the novel extraction method. Thus, the oil obtained by UAE showed greater stability compared to the oil extracted via the conventional method. TBARS of 50UAE, 25UAE, and 10UAE were 1.95, 1.77, and 1.55 mg MDA ·kg oil⁻¹, respectively were lower than CFO, 2.11 mg MDA ·kg oil⁻¹ (see Table 2.3). After extraction of AX with previously mentioned methods, UAE using 25 g of CB and 25 g flaxseed oil (25UAE) was determined to be the best quantities for the apparatus to effectively extract astaxanthin efficiently and with minimal oxidative impact. Even though the lowest lipid oxidation, POV and TBARS, were observed with 10UAE, it was not selected for future work due to its astaxanthin yield being lower than that of 25UAE. Lower POV and TBARS values were also seen with 25UAE than with FO and conventionally extracted FOSAX.

In addition to UAE reducing the potential degradative effects of using an increased temperature for extracting AX, it also had a greater yield of AX from CB. This may also be attributed, in part, to the freeze-drying step of the process, resulting in subsequent breakage of astaxanthin-protein linkages as mentioned by H.-M. Chen (1981). Additionally, the UAE

processing treatment of CB with ultrasound energy using the processing chamber facilitated AX release from the shell more than with using the conventional extraction method, similarly to the process of releasing biologically active compounds from bacterial cells used by (Mohideen et al., 2015).

During the accelerated oxidation of the oil samples at 65 °C, the POV increased linearly with heating the samples containing AX (see Figure 2.17). The linear regression coefficients were above 0.92 for the heated samples, and 0.7159 for FO at room temperature. Furthermore, a slower increase in oxidation is seen by the slopes of POV time curves for samples containing AX being below that of HFO (slope = 0.4086). The slopes of the POV time curves were 0.1508 for FOCAXUAE at 65 °C, 0.1851 for FOCAX at 65 °C, and 0.2401 for FOCAX at 65 °C are all lower than HFO. Of the heated oils containing AX, FOCAXUAE and FOCAX had the lowest POV after 1, 2, 3, and 4 hours of storage at 65 °C. The function of astaxanthin as an antioxidant is protecting the FA within the flaxseed oil by scavenging the ROS and by stalling the propagation phase.

The POV and FFA analysis of an oil are an indication of how good or bad the oil is at the present time. FFAs are also responsible for the acidity of oil. Increases in FFA content are mainly correlated to hydrolytic reactions in the oil that release glycerol and fatty acids. These reactions can be attributed to the moisture present in the oil. Compared to a study extracting astaxanthin from krill by Ali-Nehari et al. (2012), the oil used in the extraction methods here at relatively lower (25 °C for UAE) and higher (60 °C for conventional extraction) temperatures in this study had lower FFA content, thus having a lower a_w and moisture content, as opposed to oil exposed to slightly higher temperatures (35 °C, 40 °C, and 45 °C).

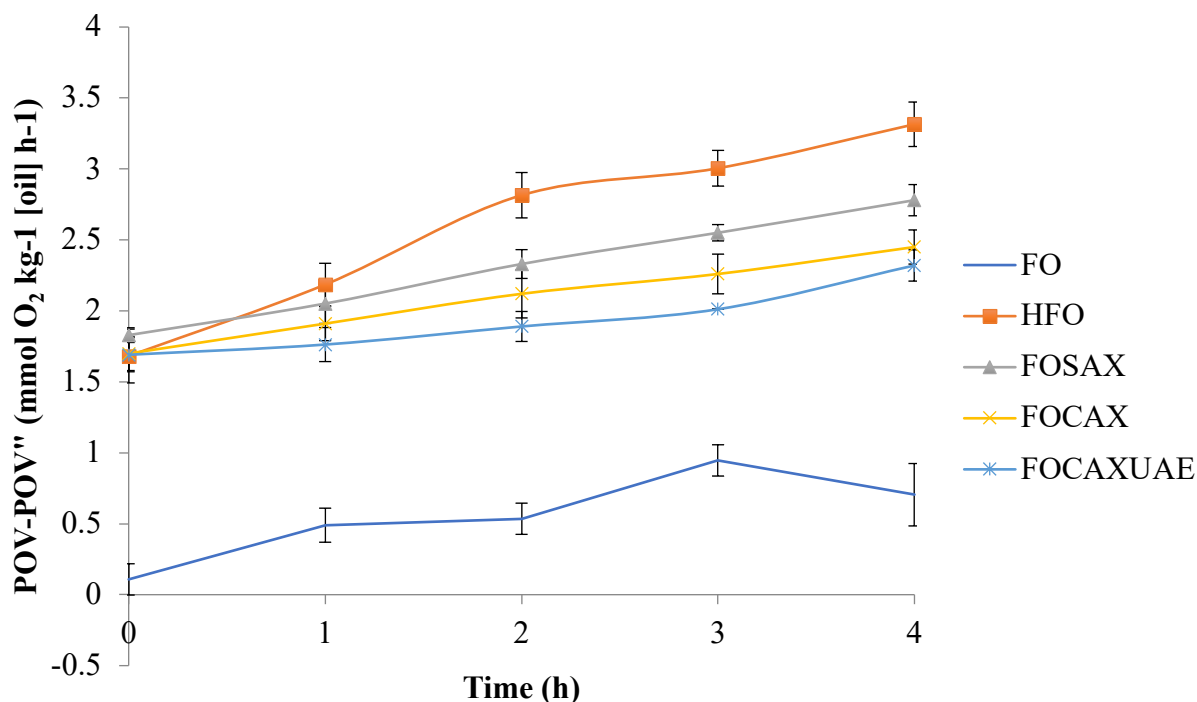


Figure 2.17. Peroxide values FO, HFO, FOCAX, FOSAX, and FOCAXUAE. FOCAX = Flaxseed oil containing crawfish astaxanthin heated at 65° C for 0-4 h; FOSAX = Flaxseed oil containing shrimp astaxanthin heated at 65° C for 0-4 h; FOCAXUAE = Flaxseed oil containing crawfish astaxanthin from ultrasound assisted extraction heated at 65° C for 0-4 h; HFO = Flaxseed oil heated at 65° C for 0-4 h; FO = Flaxseed oil not heated for 0-4 h.

Table 2.5 Slope, y intercept, and correlation coefficients for linear regression of POV of Oils not heated and heated at 65 °C vs. time.

Oil sample	Slope	y Intercept	Linear regression coefficient
FO	0.165	0.2267	0.7159
HFO	0.4086	1.7832	0.9615
FOSAX	0.2401	1.832	0.9986
FOCAX	0.1851	1.7177	0.9952
FOCAXUAE	0.1508	1.6335	0.9257

FOSAX = Flaxseed oil containing astaxanthin heated at 65° C; FOCAX = Flaxseed oil containing astaxanthin heated at 65° C; FOCAXUAE = Flaxseed oil containing crawfish astaxanthin from ultrasound assisted extraction heated at 65° C; HFO = Flaxseed oil heated at 65° C; FO = Flaxseed oil at room temperature.

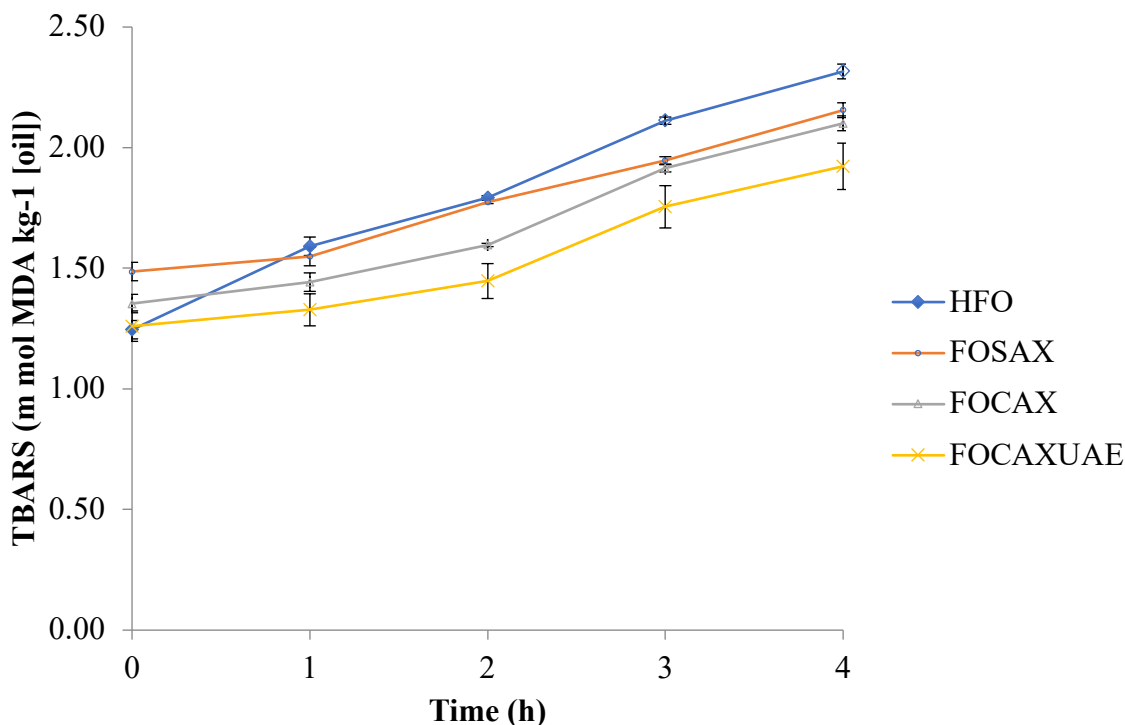


Figure 2.18. Thiobarbituric acid reactive substances of HFO, FOCAX, FOSAX, and FOCAXUAE samples at 65 °C. FOCAX = Flaxseed oil containing crawfish astaxanthin heated at 65° C for 0-4 h; FOSAX = Flaxseed oil containing shrimp astaxanthin heated at 65° C for 0-4 h; FOCAXUAE = 25 g Flaxseed oil containing crawfish astaxanthin from ultrasound assisted extraction heated at 65° C for 0-4 h; HFO = Flaxseed oil heated at 65° C for 0-4 h.

2.3.5 DPPH (2,2 diphenyl-1-picryl-hydrazyl-hydrate) radical scavenging activity

Antioxidant activity of AX extracts were determined by DPPH radical-scavenging assay (Roy et al., 2010). DPPH, a stable free radical at room temperature, has been used to evaluate the effectiveness of various antioxidant substances at free radical scavenging. The ability of astaxanthin extracts to inhibit the DPPH reaction was expressed in DPPH• inhibition (%) as shown in Figure 2.18. The decolorization of DPPH solution from purple to a yellow-colored compound (Reaction 2.1.), which is dependent upon the extent of hydrogen-donating ability during the reaction, is related to the decrease in the absorbance at 517 nm (Nagarajan, Rani, Narayanasamy, & Masthan, 2019).



The higher the DPPH scavenging (% inhibition), the higher the antioxidant activity of the sample. The FOCAXUAE sample had the greatest DPPH• scavenging ability, of all the samples and concentrations used and exhibited a higher DPPH scavenging activity than that of the control throughout the assay. At the end of the period, all the samples, except for the control (FO) showed scavenging activity greater than 50%. The results are in agreement with data reported by Taksima, Limpawattana, and Klaypradit (2015), supporting that AX functions as an antioxidant in donating H to terminate the chain reaction in lipid oxidation. Other researches have also reported that astaxanthin has a primary antioxidant function by donating electrons to unpaired electrons to neutralize free radicals or abstracting an unpaired electron which also can neutralize a radical (Kidd, 2011). The propagation step of lipid peroxidation is inhibited by the interaction of AX with free radicals and with oxygen.

The scavenging effect of AX extracts and standard FO on the DPPH• decreased in the order of FOCAXUAE (1.9 mg·g⁻¹ oil), FOCAX (0.92 mg·g⁻¹ oil), FOSAX (0.54 mg·g⁻¹ oil), FO (0.0 mg·g⁻¹ oil) equivalent to ascorbic acid (20 mg·100 ml⁻¹ ethanol), 85.85, 82.97, 82.82, and 70.85%, respectively.

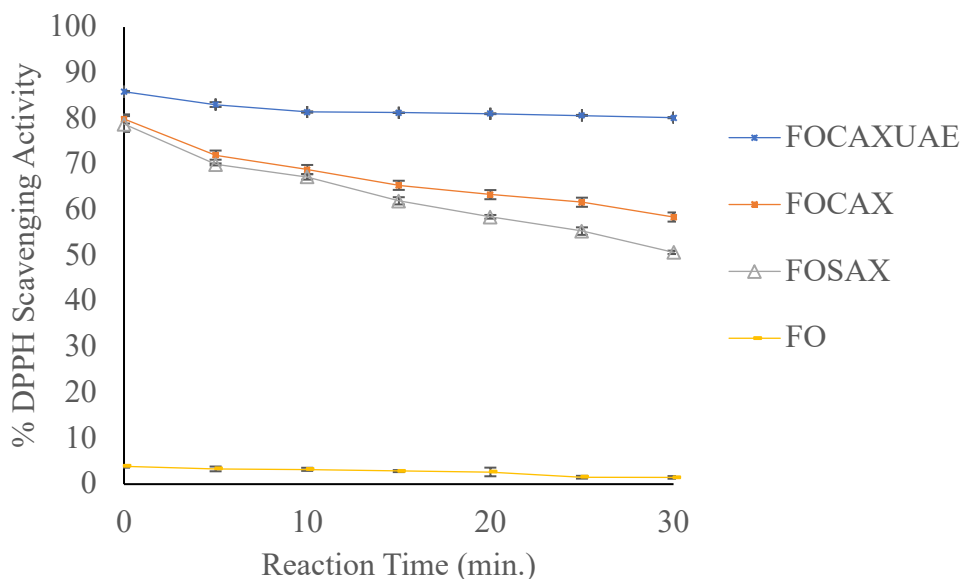


Figure 2.19 Antioxidant activity of different astaxanthin extracts in flaxseed oil determined by DPPH scavenging activity. FOCAX = flaxseed oil containing astaxanthin (0.92 mg/g) conventionally extracted from crawfish processing byproducts (n = 3). FOSAX = flaxseed oil containing astaxanthin (0.54 mg/g) conventionally extracted from shrimp processing byproducts (n = 3). FOCAXUAE = 25 g flaxseed oil containing astaxanthin (1.9 mg/g) from ultrasound assisted extraction of crawfish processing byproducts (n = 3). FO = flaxseed oil. Error bars indicate standard deviations.

For the reduction of DPPH, the standard curve was linear between 0–20 mg of ascorbic acid/100 mL. The absorbance response (y) of vitamin C ($y = -0.039x + 0.0683$, $r^2 = 0.9785$) concentrations was linear, having a strong coefficient of determination. The activity of three different extracts and the control was determined, with all tests performed in triplicate. The greatest DPPH• inhibition % after 30 minutes was with FOCAXUAE, 80.18 ± 1.02 , with FOCAX and FOSAX behind, 58.44 ± 0.22 and 50.71 ± 0.33 , respectively. The differences in antioxidative activity between extracts were mostly due to the slightly differing concentrations of AX present. Additionally, the potential isomerization of the astaxanthin molecule contributes to the increased DPPH• scavenging activity. S. Yang et al. (2015) reported that in 9-cis-astaxanthin has a greater antioxidant activity than 13-cis-astaxanthin, and the predominant naturally found, all-trans-astaxanthin. According to S. Yang et al. (2015), thermal processing has been found to induce the

formation of 9-cis-astaxanthin. Recall, that the crawfish processing byproducts had been thermally processed (parboiled) before the analyses.

2.4 Conclusion

Preliminary investigations of total extractable AX using solvents revealed that crawfish processing byproducts consisted of more astaxanthin than shrimp processing byproducts. Therefore, future investigations will focus on crawfish processing byproducts. Flaxseed oil containing astaxanthin (FOAX) derived from crawfish processing byproducts (CB) or shrimp processing byproducts (SB) were produced by extraction with two different techniques. The conventional method used to produce FOAX required more time and used a larger quantity of a valuable solvent, flaxseed oil (FO). A novel method was designed, developed and tested that produced greater yields of astaxanthin than conventional methods. The novel method employs the use of ultrasonication and ethanol combined with FO in a continuous system. The different extraction methods and types of processing byproducts showed significant differences in the amount of AX extracted. Even pre-treatment improved the recovery of extractable astaxanthin. Previous studies suggest similar end results (H.-M. Chen & S. P. Meyers, 1982). The FOAX created in all extraction methods had a specific gravity similar to the control FO. Color data revealed a significant relationship between a^* and AC. Color data did not reveal any other significant information as it pertained to any quality parameters evaluated. FOAX from CB by ultrasound assisted extraction (UAE) had a significantly lower moisture content than the other FOAX extracts. The FOCAXUAE exhibited a greater oxidative stability than other FOAX extracts, FOCAX or FOSAX, by conventional extraction. Additionally, astaxanthin recovery using the combined ethanol and flaxseed oil UAE method was more efficient than conventional extraction method, recovering more than twice the amount of astaxanthin. Compared to other

extraction methods considered, the utilization of ultrasound sonication combined with ethanol and flaxseed oil in a closed system proved to be capable of improving the extraction efficiency of astaxanthin (w/w). and minimizing the lipid oxidation significantly compared to CFO. Additionally, the antioxidant capacity was greatest with the utilization of freeze-dried parboiled CB for UAE.

CHAPTER 3 DEVELOPING ENTRAPPED FLAXSEED OIL CONTAINING CRAWFISH ASTAXANTHIN IN A PECTIN-GELATIN MATRIX GUMMY

3.1 Introduction

The increasing public perception of the correlation between diet and human health, has caused food manufacturers to develop various foods with health-promoting/protective compounds. Many of those compounds are dietary carotenoids (antioxidants), nutrients that impart protective effects on human health. Astaxanthin (AX) is a natural pigment that imparts red color to microalgae, salmon, trout, shrimp, crawfish, and flamingos. Moreover, AX has been shown to reduce the risk of developing cardiovascular disease, age-related macular degeneration, and breast cancer (Fassett & Coombes, 2012; Grimmig, Kim, Nash, Bickford, & Shytle, 2017; Inoue et al., 2017; Satoh, 2016).

The efficiency of carotenoids absorption (bioaccessibility) may limit their biological actions. Carotenoids are highly lipophilic molecules and must be incorporated within delivery systems to overcome physiological or physicochemical challenges that limit their efficacy, such as an unsuitable matrix, which directly impacts oral bioavailability, and chemical, or biochemical instability; and poor water solubility (McClements & Li, 2010). Emulsion-based delivery systems are one of the most suitable candidates for entrapping and delivering lipophilic biologically active compounds and they are relatively simple and economical to produce (Z. Zhang, Zhang, Chen, Tong, & McClements, 2015). Examples of such delivery systems include nanofibers, filled hydrogel particles, and colloidal suspensions such as emulsions, microemulsions, nanoemulsions, and multiple emulsions.

The consumption of dietary carotenoids is reported to have an inverse relationship with the risk of major clinical diseases, such as cancer. Dietary carotenoids have health benefits that

are associated with their bioavailability and metabolic fate. However, due to the lipophilic nature of astaxanthin and poor solubility in water, it has a low bioavailability. Therefore, a vehicle for effective delivery of astaxanthin is needed. Delivery of bioactive compounds to a right place at a right time or controlled release of a compound is one of the important roles of encapsulation. Encapsulation for controlled release could improve the bioaccessibility of biologically active compounds, increase the application range of food ingredients and ensure effective dosing (Desai & Park, 2005; Lu, 2017; Trojer, Nordstierna, Nordin, Nydén, & Holmberg, 2013). A specific barrier formed by encapsulation manipulates release of active compounds to provide functional benefits and unique sensory properties (Lakkis, 2007).

Hydrogels formed by cross-linkages between hydrophilic polymeric chains may be natural or synthetic (Matalanis, Decker, & McClements, 2012; Neves et al., 2015). The structural integrity of a hydrogel depends on the crosslinks formed between polymeric chains, via different types of physical interactions of chemical bonds (Neves et al., 2015). Typically, a filled hydrogel contains oil droplets trapped within the biopolymer network (Z. Zhang et al., 2015). Gelatin may have a very glossy surface due to the amount of regular reflection from the surface being high and the amount of diffuse low. Conversely, gummies containing HPMC have visually matte surfaces with greater amount of diffuse reflection, suggesting a more irregular surface.

Astaxanthin has gained much medical attention due to its health protective benefits. Astaxanthin, is a naturally occurring antioxidant that can neutralize unstable free radicals that are linked with the development of a number of diseases such as cancer, cardiovascular disease and age conditions such as Alzheimer's and macular degeneration. The biological activity of astaxanthin is highly dependent on intestinal absorption, and is why the food matrix or other delivery system is important. Several delivery systems have been developed as carriers for

astaxanthin to protect its biological activity from environmental factors and enhance its stability. Such delivery systems include nanodispersions, microencapsulations, and lipid carriers (Hama et al., 2012; I Higuera-Ciapara, Felix-Valenzuela, Goycoolea, & Argüelles-Monal, 2004; Xiaojuan Liu et al., 2018; Shen, Zhao, Lu, & Guo, 2018; Tamjidi, Shahedi, Varshosaz, & Nasirpour, 2014, 2018). Previous work has also investigated the encapsulation of a lipid extract containing astaxanthin by complex coacervation using gelatin and cashew gum (Gomez-Estaca, Comunian, Montero, Ferro-Furtado, & Fávaro-Trindade, 2016).

In the present work, the efficiency of lm-pectin-gelatin and hm-pectin-gelatin coacervation systems with and with hydroxypropyl methylcellulose (HPMC) to encapsulate core material (FOCAXUAE) were compared. Qi et al. (2013) reported a key interaction between bile salts in the gut environment and HPMC that resulted in dissolution and subsequent absorption of water-soluble drugs. The inclusion of HPMC into the delivery system contributed to micellarization and intestinal absorption of astaxanthin. The loading efficiency and release characteristics of the two coacervation systems (with and without HPMC) were considered.

3.2 Materials and Methods

Gelatin solutions were prepared by hydrating type A gelatin (Knox Original Gelatine, Unflavored, Kraft Foods, Northfield, IL), purchased from a local grocery store, in distilled water. Pectin solutions were prepared separately with high-methoxyl pectin (Pre-Hydrated® HM Rapid Powder, HMP, Lot # 43621, DE>50%, 181 kDa mw) and low-methoxyl pectin (TIC Pretested® Pectin LM 32 Powder, LMP, Lot # 571091, 32% DE, 130 kDa mw), both kindly provided by TIC Gums (Belcamp, MD), to distilled water with agitation overnight to ensure complete hydration. Sunflower lecithin powder (lecithin, see Table 3.1 for lipid content) was purchased from Now Foods (Bloomington, IL). Hydroxypropyl methylcellulose (HPMC, MW ~10 kDa,

methoxyl content ~28 wt. %, hydroxypropoxy content ~9.5 wt. %, equivalent to Methocel E, viscosity grade 60 cP, Alfa Aesar, Germany), and was purchased from Fisher Scientific. Ethanol (190 proof), chloroform, and methanol were all analytical grade solvents.

Sunflower lecithin (5% wt. of FOCAXUAE wt.) was used as emulsifier to prepare the oil phase containing sodium deoxycholate (1% wt. of FOCAXUAE). This oil phase was manually distributed into the aqueous phase to an oil fraction of 32 g/ 26 g of pectin solution (10% wt.). The coarse O/W emulsions obtained were sonicated using an ultrasound processor (Cole Palmer) in a dry ice, ethanol slurry under a nitrogen flux to prevent thermally induced lipid oxidation.

High-methoxyl apple pectin (HMP) and low-methoxyl pectin (LMP) were kindly provided by TIC Gums (Belcamp, MD). Glucose syrup (Karo Light corn syrup, ACH Food Companies, Inc., Oakbrook Terrace, IL, USA) was purchased from Walmart.

Table 3.1. Phospholipid content in sunflower-lecithin.

Naturally occurring phospholipids (per serving) (10 g):	
Phosphatidyl Choline	2.5 g
Phosphatidyl Inositol	1.8 g
Phosphatidyl Ethanolamine	1.1 g

3.2.1 Preparation of FOCAXUAE for gummy fabrication

A stock FOCAXUAE was prepared by concentrating (5) subsequent FOCAXUAE extractions with the same FO and E ratio as previously described in section 2.2.3.2. of Chapter 2, with slight modification. To effectively concentrate astaxanthin, crawfish processing byproducts (CB) were replaced with a new batch of CB after each 10-minute ultrasound assisted extraction cycle. Following the extraction period, the CB were washed with ethanol until clear. Then the

filtrate was pooled and complete evaporation of the ethanol was accomplished by using a rotary evaporator (Büchi Rotavapor R-114, Büchi Labortechnik AG, Flawil, Switzerland). This produced a concentrated FOCAXUAE (35 ml).

3.2.2 Preparation of Emulsions

Oil in water emulsions with desired concentrations of low- or high-methoxyl pectin containing concentrated FOCAXUAE were prepared by a method of Couëdelo et al. (2010) at room temperature with slight modification. The aqueous phase, emulsifier solution was prepared by first dissolving HMP or LMP in distilled water overnight to allow complete hydration, followed by the subsequent addition of lecithin (20% wt. of oil). The amount of FOCAXUAE in the O/W emulsion was adjusted so that each gummy contained approximately 10% FOCAXUAE of the total weight of the gummy after setting. Emulsions prepared with HPMC, were prepared without lecithin.

3.2.2.1 Emulsion Stability

The stability of the emulsions was assessed according to a method described by Mohideen (2011) with modifications. Emulsion samples (3 g each) were placed in 13 x 100 mm disposable borosilicate glass culture tubes (Kimble, Vineland, NJ) in a tube rack on a benchtop at room temperature (25±0.5 °C) for 2 days. Next the samples were centrifuged at 1163 x g for 30 minutes (BD-Clay Adams Compact II centrifuge), and the amount of emulsion cream separated was measured. To determine the emulsion stability, or inability of phases to separate, the percent of oil to separate from the emulsion must be calculated. The oil recovery percentage was calculated using equation 3.1. The emulsion stability (ES) was determined using equation 3.2.

$$\text{Oil recovery (OR) \%} = \frac{\text{weight of oil recovered}}{\text{Emulsion weight (initial)}} \times 100 \quad (\text{Eq. 3.1})$$

where oil separated is the weight of the oil separated from the cream layer after centrifugation treatment.

$$\text{Emulsion Stability (ES)} = 100 \% - \text{OR}\% \quad (\text{Eq. 3.2})$$

3.2.3 Formulation and production of pectin-gelatin delivery system

Pectin-gelatin delivery system (PGDS) were prepared using the method of DeMars and Ziegler (2001) with modifications. Table 3.2 shows the PGDS recipes. Figure 3.2 is a flow diagram for the preparation of the gummies. Each batch was made using the same amounts for sugar slurries (brought to 240 g by addition of distilled water). O/W emulsions were prepared such that when added to gelling slurries (gelatin, ~15-30 g) and the desired amount of gelatin and pectin were achieved. Gelling slurries were formulated such that the final PGDS contained the desired concentration of pectin and gelatin to facilitate associative interactions without precipitation of either. Additionally, for a confectionery gummy production, it is essential to include glucose syrup in proportional amounts to sugar (1:1) to prevent crystallization of the sugar. In the present work, the word “gummy” and “PGDS” are used interchangeably.

After both solutions (gelling slurry and sugar slurry) were mixed under constant stirring for 10 minutes, the homogenous viscous solutions obtained were poured into 160 square cavity silicone molds. Each resulting gummy cube had a volume of 1 cm³ (quantity of solutions deposited in the mold were estimated to be the same for all gummies when pouring). Once the trays filled, they were placed in the refrigerator for 48 hours to achieve gel consistency. Each gummy candy weighed 1±0.03 g. After gelling they were removed from the molds and lightly dusted with powdered sugar to prevent them from sticking. They were then placed in square 5.5 in. x 5.5 in. weigh dishes to dry at room temperature (25±0.5 °C) for another 48 h. For release

PGDS cubes were placed in separate conical tubes (50 ml) and refrigerated. PGDS with FO in the emulsion preparation step was prepared as a blank.

Table 3.2. Design and development of pectin-gelatin gummy pieces.

Ingredients (g)	PGDS1 (pH 3.55)	PGDS2 (pH 2.85)	PGDS3 (pH 2.8)	PGDS4 (pH 2.8)	PGDS5 (pH 2.9)	PGDS6 (pH 2.9)	PGDS7 (pH 2.9)
Sucrose**	101	111	101	111	111	102	102
Corn syrup**	91	103	91	103	103	90	90
H ₂ O**	100	100	100	120	120	50	50
LMP*		2		4		3	
HMP*	4		2		4		3
HPMC*						5% w/w	5% w/w
dH ₂ O*						60	60
Gelatin***	15	30	30	30	30	15	15
H ₂ O***	30	30	30	30	30	20	20
FOCAXUAE*	30	20	20	30	32	32	32
Tween 20		0.27	0.4	0.35	0.4	0.27	0.27
Ascorbic acid	2	2.5	2.7	11.46	3	6	7

Note: PGDS1- PGDS7 are prepared with pectin (LMP or HMP), with or without HPMC, FOCAXUAE, and distilled (d) H₂O*. Sucrose (table sugar), corn syrup (Karo®, ACH Food Companies, Inc., Oakbrook Terrace, Il.)), and dH₂O**. Type-A gelatin hydrated with dH₂O*** becomes the gelling solution*** Tween 20 was added during the mixing of the three final solutions followed by lowering the pH with 50% food grade citric acid solution. HPMC is 5% of final emulsion (g). The ingredient ratios of PGDS1 were not capable of forming the desired delivery system, thus no further work was done on PGDS1. ^{2*}

^{2*} denotes the emulsion containing pectin, dH₂O, and FOCAXUAE. ** denotes the sugar slurry, *** denotes the gelling slurry.

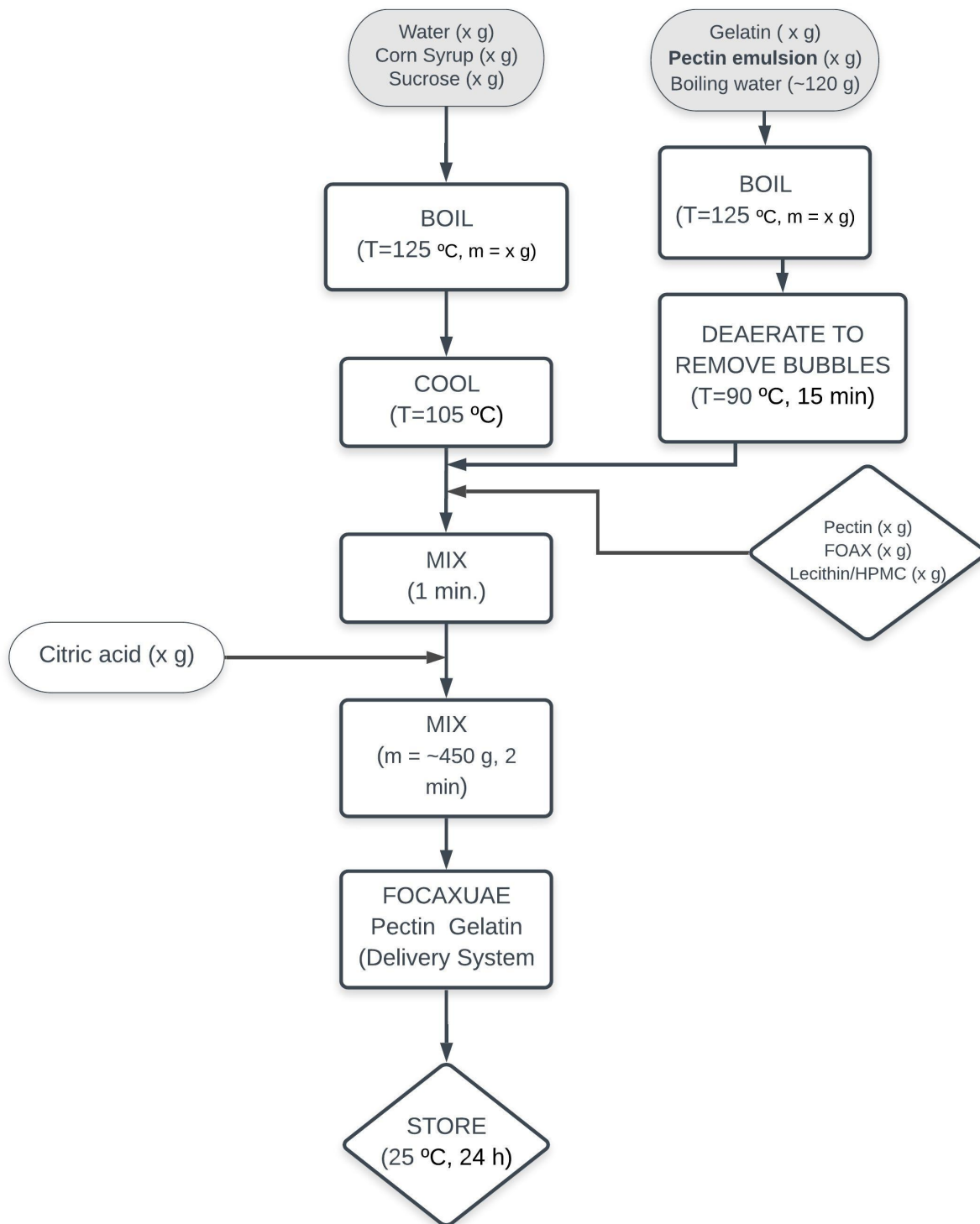


Figure 3.1. Flow diagram for preparation of FOCAXUAE pectin gelatin delivery system. To promote coacervation, the pH was changed to $\sim 3 \pm .5$ with citric acid (50% v/v food grade citric acid and distilled water) at 25 ± 0.5 °C under a stirring rate of 600 rpm for 30 min to achieve uniform mixing, complete complex coacervation, and minimize aeration. Source: Muhoza, Xia, and Zhang (2019).

3.2.4 Water Activity and soluble solids content of pectin gelatin cubes

After PGDS fabrication, their water activity (a_w) were evaluated. The a_w of samples was determined using a Pawkit a_w meter (AquaLab Pawkit, Decagon Devices Inc., Pullman, WA) at 25 ± 0.5 °C. To determine total soluble solids (TSS) of the PGDS, distilled water (DW) was combined with macerated PGDS (1:1 g/g). TSS was then determined from the mixture by refractometry, using a digital refractometer (AR200, Reichert Analytical Instruments, Depew, NY, USA) with values expressed in Brix (°Brix). All analyses were carried out in triplicate.

3.2.5 Determination of FOCAXUAE content in PGDS

Using a modified method of Blighs and Dyer (1959) for extracting total lipids, the astaxanthin content in the PGDS was determined. To quantify the mass (g) of total lipid initially loaded in a PGDS, a 50 mL tube containing macerated PGDS ($5 \text{ g} \pm 0.1$) and 2.5 mL DW was added. Then a ternary solvent system was created as follows: for each gram of sample, 3.75 mL of 1:2 CHCl_3 : MeOH was added and vortexed (1000 rpm) until complete dissolution had occurred or for 90 seconds. Then an additional two mL of DW was mixed with the sample and vortexed a second time (30 seconds). The alcoholic layer containing non-lipids (top layer) was carefully removed by aspiration using a syringe and the CHCl_3 fraction (bottom layer) containing the lipids was added to a tared vial. The solvent was evaporated from the tared vial using an analytical evaporator until constant weight was achieved. The cooled vial was weighed, and the weight of the lipid residue recorded. Triplicate determinations were performed for each sample. The average values were recorded as, Total FOCAXUAE content (TFC) in PGDS.

3.2.6 Determination of lipid released and astaxanthin content in PGDS

The amount of lipid released was determined in the same manner as TFC, except that the released material from the gummies was used instead of macerated gummies. The amount of

astaxanthin in the gummy was proportional to amount of oil quantified per gummy. Three PGDS samples were weighed (data recorded as initial PGDS weight) and placed inside 50 mL conical tubes for dissolution studies. re placed on a tared balance and the weights recorded. For quantification of lipid released, 11.25 mL of 1:2 CHCL₃: MeOH were added to each 50 mL conical tube containing PGDS samples and 10mL of dissolution media and vortexed (1000 rpm) for 60 seconds. The alcoholic layer containing non-lipids (top layer) was carefully removed by aspiration using a syringe and the CHCL₃ fraction (bottom layer) containing the lipids was added to a tared vial. The solvent was evaporated from the tared vial using an analytical evaporator until constant weight was achieved. The cooled vial was weighed, and the weight of the lipid residue recorded. Triplicate determinations were performed at each designated time. The averaged values were recorded as lipid released.

Astaxanthin content of total lipid extracted from the gummy was measured using to the method of Saito and Regier (1971) with a slight modification. Following “Determination of FOCAXUAE content in PGDS” (see section 3.2.5.) and the determination of lipid released in PGDS in this section, 30 mg of recovered oil was mixed with 10 ml of petroleum ether (PE) and allowed to stand for 30 min. The absorbance of oil, appropriately diluted, was measured at 468 nm using a GENESYS 20 UV-Vis spectrophotometer (Thermo Fisher Scientific, Inc., Rochester, NY, USA). The concentration (C) of astaxanthin in the PGDS was calculated using the equation given by Saito and Regier (1971) with a slight modification as follows:

$$AXC (\mu g/g \text{ lipid}) = \frac{A_{468} \times \text{volume of PE} \times \text{dilution factor}}{0.2 \times \text{weight of sample used in gram}}$$

(Eq. 3.3) where 0.2 is the A₄₆₈ of 1 µg/ml standard astaxanthin and 1 is the dilution factor of the sample.

3.2.7 Swelling and Release media

In order to examine the potential of the pectin-gelatin delivery system (PGDS) gummy as a delivery system for bioactive compounds, release characteristics of PGDS with entrapped FOCAXUAE were investigated in release media. The gastrointestinal (GI) tract environment was modeled with two simulated digestive fluids as described by Chotiko and Sathivel (2017) and Taylor et al. (2019) with some modifications. In accordance with the United States Pharmacopeia, simulated gastric fluid (SGF at pH 1.2, without pepsin) consisted of 2 g of sodium chloride (Fisher Scientific Inc., Pittsburgh, PA), 7 mL of hydrochloric acid (Fisher Scientific Inc., Pittsburgh, PA), and distilled water used to adjust the total volume to 1 L. For simulated intestinal fluid (SIF at pH 6.8, without enzyme), 6.805 g of monobasic potassium phosphate (Sigma Aldrich, St. Louis, MO) and 0.896 g of sodium hydroxide (Fisher Scientific Inc., Pittsburgh, PA) were dissolved in distilled water and the total volume was adjusted to 1 L.

3.2.7.1 Swelling Studies

Swelling of hydrogels affects the controlled release of the compounds entrapped in the hydrogel matrix (Xiudong Liu et al., 2004). To evaluate the water sorption resistance of the PGDS with entrapped astaxanthin, three samples were weighed (W_d), 1 ± 0.03 g each and then immersed in 30 mL of dissolution media (distilled water (DW), simulated gastric fluid (SGF pH 1.2) or simulated intestinal fluid (SIF pH 6.8) at 37 °C, with shaking (50 rpm). Swollen gels were removed from dissolution media at predetermined time intervals, blotted dry, and weighed (W_f) to track sorption kinetics. The swelling index (SI) was determined as described by (Raafat, Eid, & El-Arnaouty, 2012) using the equation below. Each tube was initially prepared with equal volumes of dissolution fluids.

$$SI (\%) = \frac{W_f - W_i}{W_f} \times 100 \quad (\text{Eq. 3.4})$$

Where W_f and W_i are the weights of swollen and dry gummies, respectively

3.2.7.2 *In vitro* Release Study

Release of astaxanthin from PGDS was investigated in SGF and SIF for 120 and 180 min, respectively as described by Chotiko and Sathivel (2017) and Taylor et al. (2019) with some modifications. Three grams of PGDS were placed in 50 ml conical tubes containing 10 ml of SGF or SIF. The tubes were incubated with continuous agitation, at 50 rpm about their horizontal axis in a circular motion using an orbital shaker incubator equipped with a temperature control system (37 °C). Three individual tubes were sampled at predetermined time intervals to determine FOCAxUAE released during incubation. Astaxanthin content in the gummies was determined by the methods mentioned in sections 3.2.5 and 3.2.6 above.

The amount of lipid released was determined by the method mentioned above in section 3.2.6. With the amount of lipid released and the concentration of astaxanthin (mg) per g of oil, the bioaccessibility of AX can be determined.

$$(\%) \text{ Bioaccessibility} = \frac{\text{amount FOCAxUAE recovered from PGDS at each time (mg)}}{\text{amount of FOCAxUAE initially loaded in PGDS (mg)}} \times 100 \quad (.3.5)$$

Bioaccessibility can be defined as the theoretical amount of astaxanthin available for absorption. The amount of FOCAxUAE initially loaded per PGDS is ~200 mg per a ~2g gummy and ~100 mg per 1 g gummy, based on the method in section 3.2.5. Triplicate determinations were performed for each sample.

3.2.8. Morphology using scanning electron microscopy (FIB-SEM)

Freeze dried gummies (PGDS2, PGDS3, PGDS4, PGDS5, PGDS6, PGDS7) were mounted on aluminum scanning electron microscopy (SEM) specimen stubs and coated in an Edwards S150 sputter coater with platinum. Coated gummies were observed using a dual-beam scanning electron microscope (FEI Quanta 3D FEG FIB-SEM) instrument (Louisiana State

University, Louisiana) interfaced with EDAX EDS/EBSD system at an acceleration voltage of 5kV and 3 pA under high vacuum. SEM micrographs were used to obtain information about the surface morphology and encapsulated bioactive compound.

3.2.8 Statistical Analysis

Mean values and standard deviations of triplicate reproducible analyses are reported. The statistical significance of observed differences among treatment means was evaluated by analysis of variance (ANOVA) was carried out to determine the difference among treatments means with SAS software Version 9.4 (SAS Institute Inc., Cary, NC) using a *post hoc* Tukey's studentized range test (SAS, 2013).

3.3 Results and Discussion

3.3.1 Water activities, moisture, soluble solids, and astaxanthin content of the delivery system

Protein and polysaccharide interactions play an important role in the structure and stability of foods and food products. A key factor in fabricating novel food products is the control and manipulation of macromolecular interactions between hydrocolloid constituents. The functional properties of proteins are affected by their interactions with polysaccharides, and vice versa. Analyses were performed on delivery system to determine the moisture and water activity of the PGDS samples. Results are shown in Table 3.3. PGDS7 and PGDS6 had the lowest water activity, which may be due to the hydrophilicity of HPMC, a synthetic polymer, binding more water.

Table 3.3. Compositional parameters of pectin gelatin delivery systems (PGDS)

	Water activity	Moisture (%)	Soluble solids (°Brix)	AC (mg)
PGDS2	0.81 ± 0.011^D	25.20 ± 0.10^B	75.27 ± 0.25^C	0.27 ± 0.02^B
PGDS3	0.77 ± 0.005^B	25.03 ± 0.15^B	73.43 ± 0.02^B	0.27 ± 0.02^B
PGDS4	0.79 ± 0.006^C	26.93 ± 0.23^B	73.24 ± 0.03^B	0.38 ± 0.04^B
PGDS5	0.79 ± 0.006^C	27.00 ± 0.2^B	71.49 ± 0.03^A	0.43 ± 0.05^A
PGDS6	0.76 ± 0.015^B	20.76 ± 0.06^A	71.69 ± 0.01^A	0.54 ± 0.02^A
PGDS7	0.73 ± 0.003^A	20.95 ± 0.07^A	70.73 ± 0.02^A	0.54 ± 0.01^A

Note: ^{A-D} Means \pm SD not containing a letter in common are significantly different ($p=0.05$). Each PGDS weighs 1 ± 0.03 g. PGDS2 - 2% LMP:8% G, pH 2.85 (5%); PGDS3 - 2% HMP:8% G pH 2.8 (5%); PGDS4 - 4% LMP:30% G pH 2.8 (8%); PGDS5 - 4% HMP:30% G pH 2.9 (8%); PGDS6 - 3% LMP:15% G:5% HPMC pH 2.9 (10%); PGDS7 - 3% HMP:15% G:5% HPMC pH 2.9 (10%); AC = astaxanthin content

Developed gels potentially affect carotenoid bioaccessibility. High-DM pectin increases the lipolysis, bile salt binding, and micellarization (conversion into micelles) of polar carotenoids, resulting in increased uptake (Cervantes-Paz et al., 2017). Conversely, low-DM pectin can reduce the levels of calcium involved in the lipolysis and generation of lipid digestion products for micelle formation, thus decreasing carotenoid uptake (Devraj et al., 2013).

The admix of ingredients and setting times play an essential role in the formulation and gel strength of products. The heterogeneous structures produced by the gummy formulations can be seen in Figure 3.3., Figure 3.4 (glossy concave top surface), Figure 3.5 (glossy concave top surface), Figure 3.6, and Figure 3.7 (incompatibility of gelatin and pectin, concave structures, and surface haze, respectively), with the exception of PGDS7 and PGDS6 (Figure 3.8. and 3.9. This is explained by the lack of favorable polymer interactions in forming gel networks with different concentrations of LMP and lack of cations or the rapid gel formation of different concentrations of HMP at a pH lower than 3 and coalescence of FOCAXUAE droplets. It was reported that an increase in ionic strength of the media over 0.1 mM decreased the coacervation yield of gelatin (Burgess & Carless, 1985). The SIF had an ionic strength greater than 0.1 mM as a result of the 0.896 g of NaOH/liter (0.22 mM),

therefore further complex coacervation between gelatin and pectin was unlikely to occur. This supports the need for HPMC in developing a gel that is capable of withstanding the GI environment.

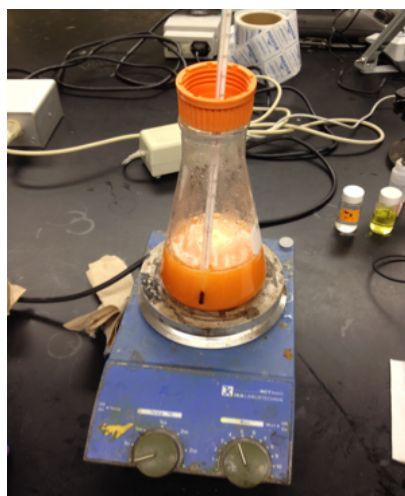


Figure 3.2. Addition of FOCAXUAE to emulsion with pectin and gelatin.

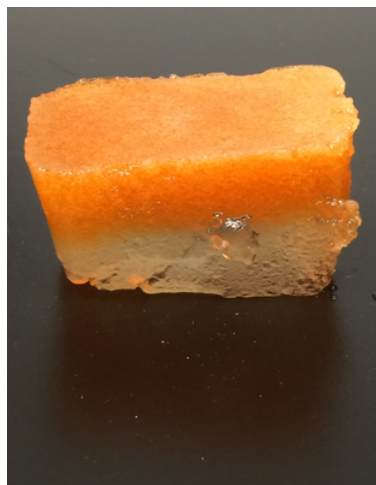


Figure 3.3. PGDS1 - 4% HMP:15% G pH 3.55 (12%).

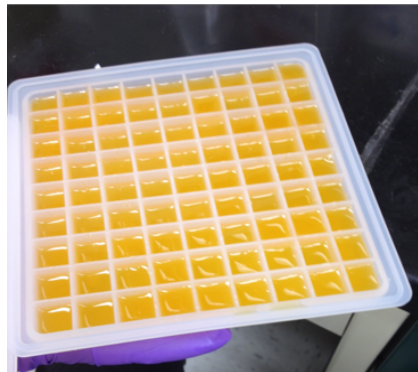


Figure 3.4. PGDS2 - 2% LMP:8% G, pH 2.85 (5%).

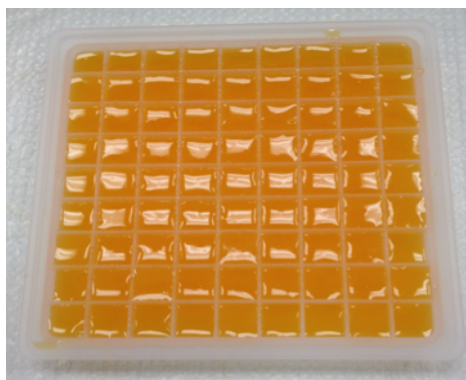


Figure 3.5. PGDS3 - 2% HMP:8% G pH 2.8 (5%).

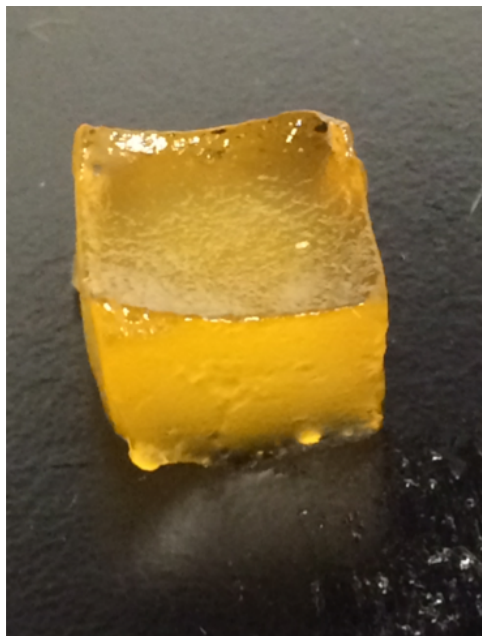


Figure 3.6. PGDS4 - 4% LMP:30% G pH 2.8 (8%).



Figure 3.7. PGDS5 - 4% HMP:30% G pH 2.9 (8%).

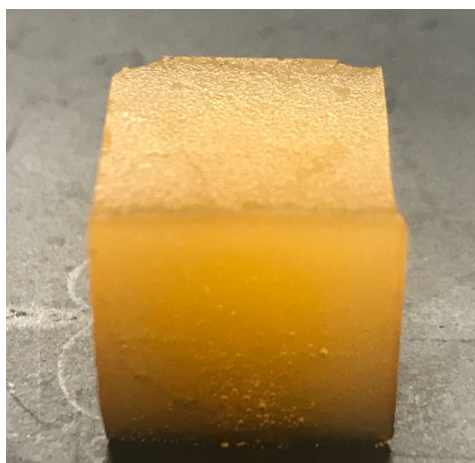


Figure 3.8. PGDS6 - 3% LMP:15% G:5% HPMC pH 2.9 (10%).

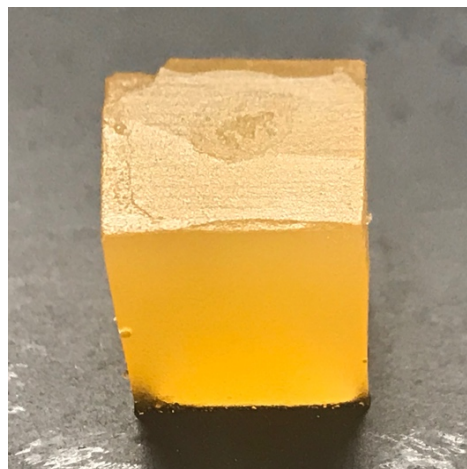


Figure 3.9. PGDS7 - 4% LMP:15% G:5% HPMC pH 3.55 (10%).

The segregative interactions among pectin and gelatin biopolymers (without a nonionic surfactant (HPMC)) are seen in Figure 3.3. The emulsion phase containing FOCAXUAE can be seen atop the translucent gelatin phase, Figure 3.3. With PGDS1, it is clear that the upper phase is rich in FOCAXUAE: pectin (reddish orange in color) while the lower is rich in gelatin (translucent). The segregation is due to the incompatibility between positively charged gelatin ($pI \sim 7$) and neutral FOCAXUAE: pectin. Phase separation due to the segregative interactions

occurs above a certain concentration of polymers at which they become thermodynamically favorable (Doublier, Garnier, Renard, & Sanchez, 2000).

3.3.2 Swelling and Release Study

According to previous research by Sriamornsak, Nunthanid, Cheewatanakornkool, and Manchun (2010), the release of a compound from a hydrogel matrix is divided into two phases, rapid release period, and a declining rate period. In some cases, the first phase would be governed by two mechanisms that dictate drug compounds release from a matrix, swelling and diffusion. The second phase, is more or less a period of steady release or erosion, rather than a declining rate, although that rate is lower than the rate of release due to swelling and diffusion. During such period, the gel swelling has reached equilibrium (Brannon-Peppas & Peppas, 1991). However, such phenomena are seen with water-soluble compounds integrated in a matrix, while for low water-soluble compounds like carotenoids, self-erosion of the matrix is the primary release mechanism.

Release of FOCAXUAE from the delivery systems was investigated in SIF and SGF. When incorporating bioactive compounds into a product for human consumption, one factor that goes into the development and design is the delivery system, which encompasses the release of the bioactive components into a targeted region to facilitate their absorption.

3.3.2.1 Swelling Study

The swelling index indicates the percentage swelling of the delivery system caused by the uptake of the dissolution media and subsequent growth. With there being no polymer erosion, the water uptake and swelling resulted in an increase in weight. Greater water uptake was seen in PGDS7 (high-methoxyl) than with PGDS6 (low-methoxyl) in SIF, SGF, and DW dissolution medias, Figure 3.10 and Figure 3.11, respectively. Water uptake between PGDS7 and PGDS6

did not differ in DW, see Figure 3.12. Swelling initially occurred, within 15 minutes, among all formulations in both SGF at pH 1.2 and SIF at pH 6.8. Swelling was most profound in SIF for PGDS7 with a SI of $126.46 \pm 5.1\%$ after 180 minutes followed by PGDS6 with a SI of $90.61 \pm 6.8\%$ after 60 minutes. This further confirms that the prepared delivery systems, PGDS6 and PGDS7, have the capability to withstand the intestinal tract and its environments. The other delivery systems failed to maintain their structure and either gelled or eroded completely and had swelling indices under 30%. The large swelling indices of HPMC gummies (PGDS7 and PGDS6) can be attributed to the hydrophilicity of HPMC.

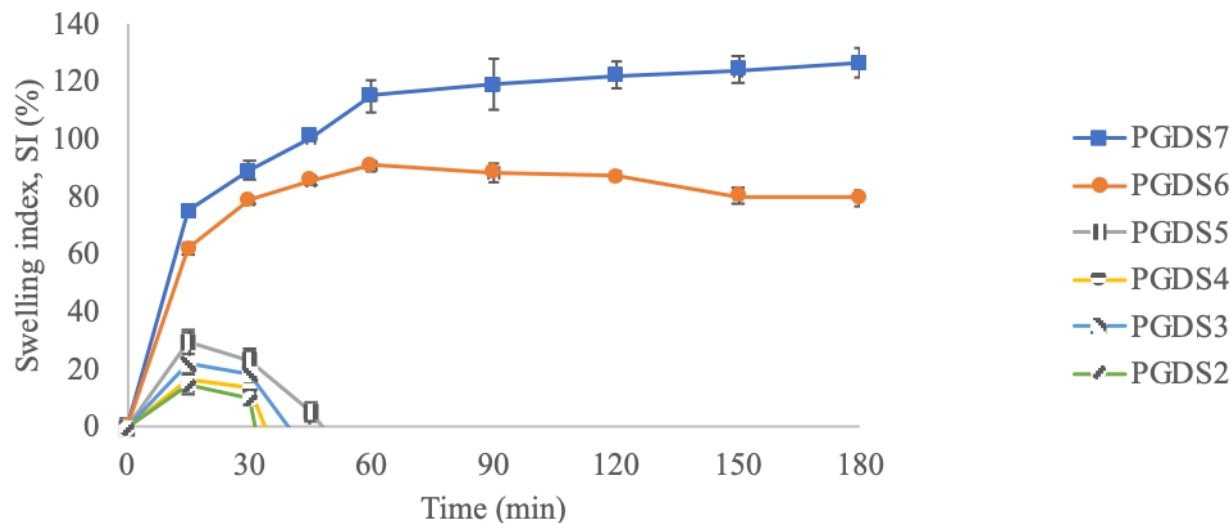


Figure 3.10. Swelling index studies of pectin-gelatin based delivery systems loaded with FOCAXUAE in simulated intestinal fluid (SIF pH 6.8). PGDS7, 3% high-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 3.55; PGDS6, 3% low-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 2.9; PGDS5, 4% high-methoxyl pectin 30% gelatin gummies pH 2.9; PGDS4, 4% low-methoxyl pectin 30% gelatin gummies pH 2.8; PGDS3, 2% high-methoxyl pectin 8% gelatin gummies pH 2.8; PGDS2, 2% low-methoxyl pectin 8% gelatin gummies pH 2.85.

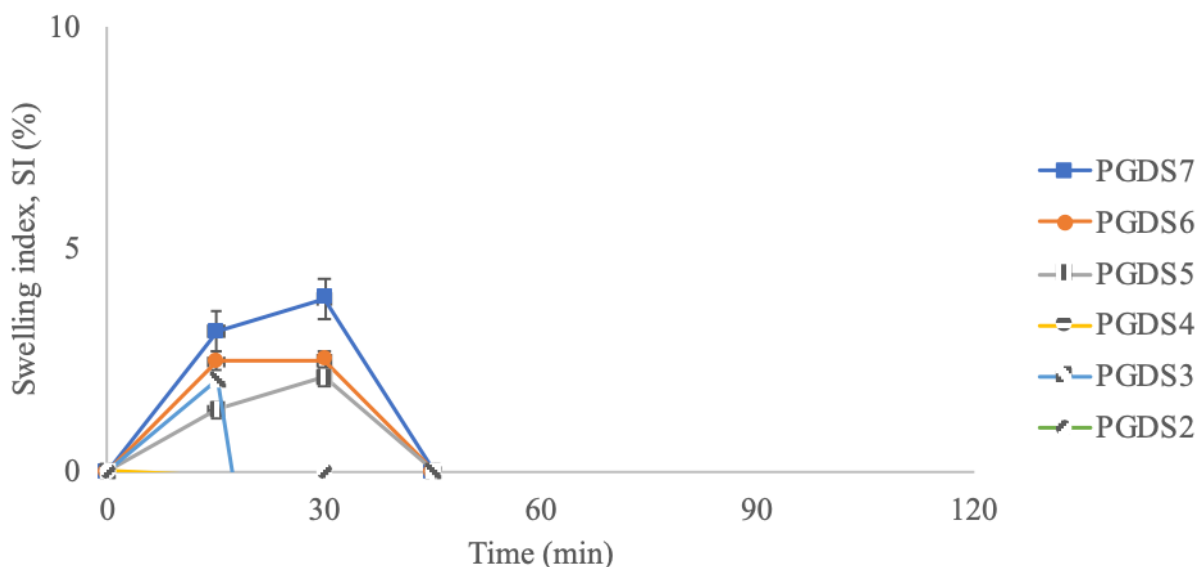


Figure 3.11. Swelling index studies of pectin-gelatin based delivery systems loaded with FOCAXUAE in simulated gastric fluid (SGF pH 1.2). PGDS7, 3% high-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 3.55; PGDS6, 3% low-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 2.9; PGDS5, 4% high-methoxyl pectin 30% gelatin gummies pH 2.9; PGDS4, 4% low-methoxyl pectin 30% gelatin gummies pH 2.8; PGDS3, 2% high-methoxyl pectin 8% gelatin gummies pH 2.8; PGDS2, 2% low-methoxyl pectin 8% gelatin gummies pH 2.85.

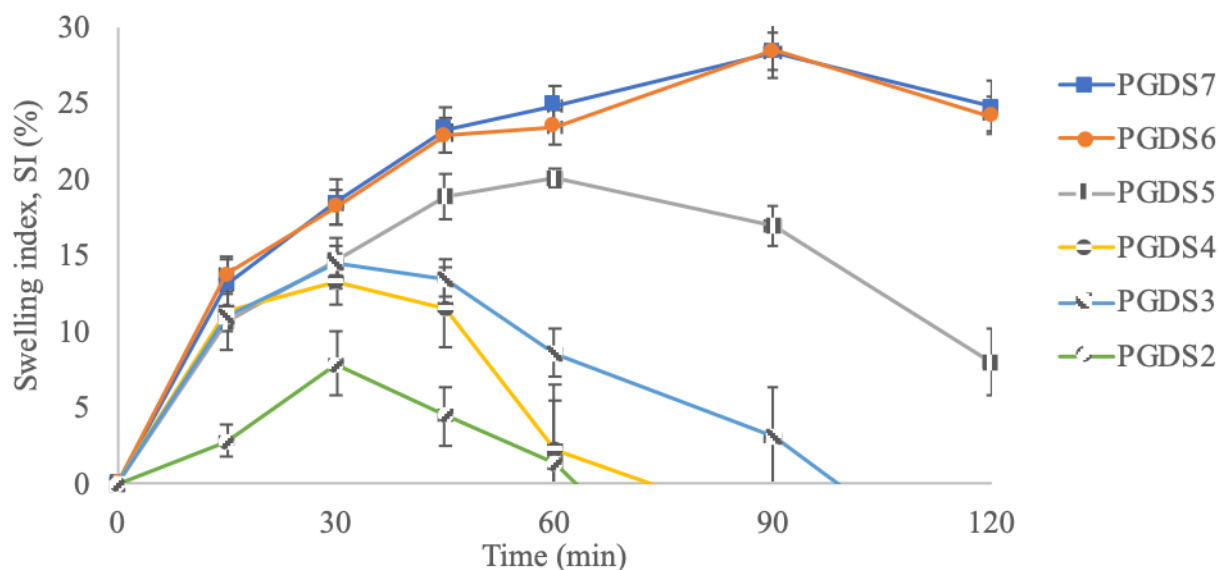


Figure 3.12. Swelling index studies of pectin-gelatin based delivery systems loaded with FOCAXUAE in distilled water (DW). PGDS7, 3% high-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 3.55; PGDS6, 3% low-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 2.9; PGDS5, 4% high-methoxyl pectin 30% gelatin gummies pH 2.9; PGDS4, 4% low-methoxyl pectin 30% gelatin gummies pH 2.8; PGDS3, 2% high-methoxyl pectin 8% gelatin gummies pH 2.8; PGDS2, 2% low-methoxyl pectin 8% gelatin gummies pH 2.85.

3.3.2.2 Releasing Study

Despite the formation of a gel due to the presence of gelatin, the absence of divalent cations in the media used to prepare the PDGS prevent LMP gummies from forming junctions and a network of polymer chains to entrap FOCAXUAE. Thus, making PGDSs formulated with LMP incapable of controlling the release of FOCAXUAE, see Figures . LMP is capable of forming three dimensional rigid and water insoluble hydrogels through ionic interactions between divalent cations (i.e., Ca^{2+}), and the free carboxyl groups of the pectin (Powell, Morris, Gidley, & Rees, 1982). PGDS formulations containing HMP were under the potential gelling conditions required (i.e., necessary concentration of pectin, pH, concentration of sucrose) during preparation. HMPs added gel forming ability was increased at lower pH (SGF, pH 1.2), as compared to the pH of SIF (pH 6.8), therefore slowing the release of astaxanthin in SGF. Gel formation of HMPs only occur when the pH value is sufficiently low to suppress the electrostatic repulsions between charged carboxyl groups ($-\text{CO}_2\text{H}$ or $-\text{COOH}$) resulting in increased hydrogen bonding and hydrophobic interactions between the pectin molecules (Löfgren & Hermansson, 2007). The same would be the case for LMP if divalent cations were present.

None of the PGDS gummies tested in SGF dissolution media were able to prevent release of FOCAXUAE into the stomach milieu mimicking solution. However, with PGDS7 and PGDS6, the acid environment of SGF dissolution media was slower to degrade the HPMC coating. The HPMC provides gastric-resistant properties, which resulted in a delayed liberation of the incorporated FOCAXUAE (Cole et al., 2002), reported similar finding using HPMC as an enteric coating. However, if the FOCAXUAE would have been encapsulated by pectin in the PGDS gummies without HPMC, early release may have been slowed or prevented. According to

the release study, gels with pectin dissolved slower in SGF than SIF. To mimic the GI tract transit times, the gels were only subjected to SGF for 120 minutes, and to SIF for 180 minutes.

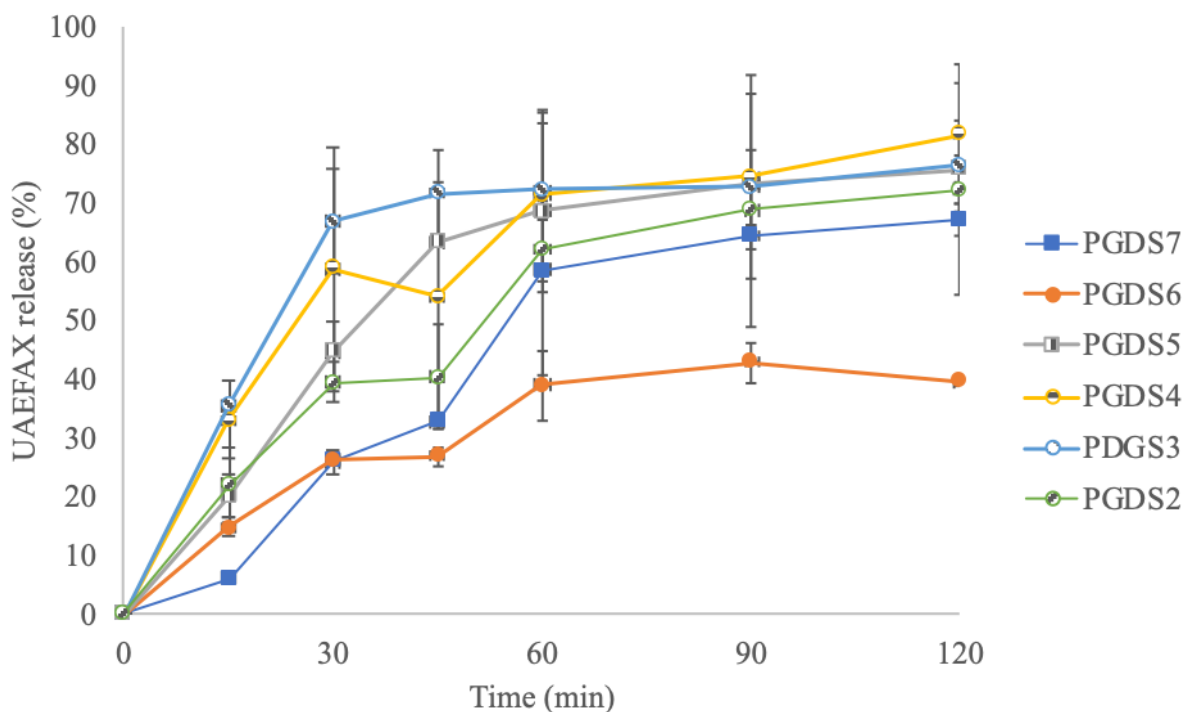


Figure 3.13. Release characteristics of pectin-gelatin based delivery systems loaded with FOAXUAE in simulated gastric fluid (SGF pH 1.2). PGDS7, 3% high-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 3.55; PGDS6, 3% low-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 2.9; PGDS5, 4% high-methoxyl pectin 30% gelatin gummies pH 2.9; PGDS4, 4% low-methoxyl pectin 30% gelatin gummies pH 2.8; PGDS3, 2% high-methoxyl pectin 8% gelatin gummies pH 2.8; PGDS2, 2% low-methoxyl pectin 8% gelatin gummies pH 2.85.

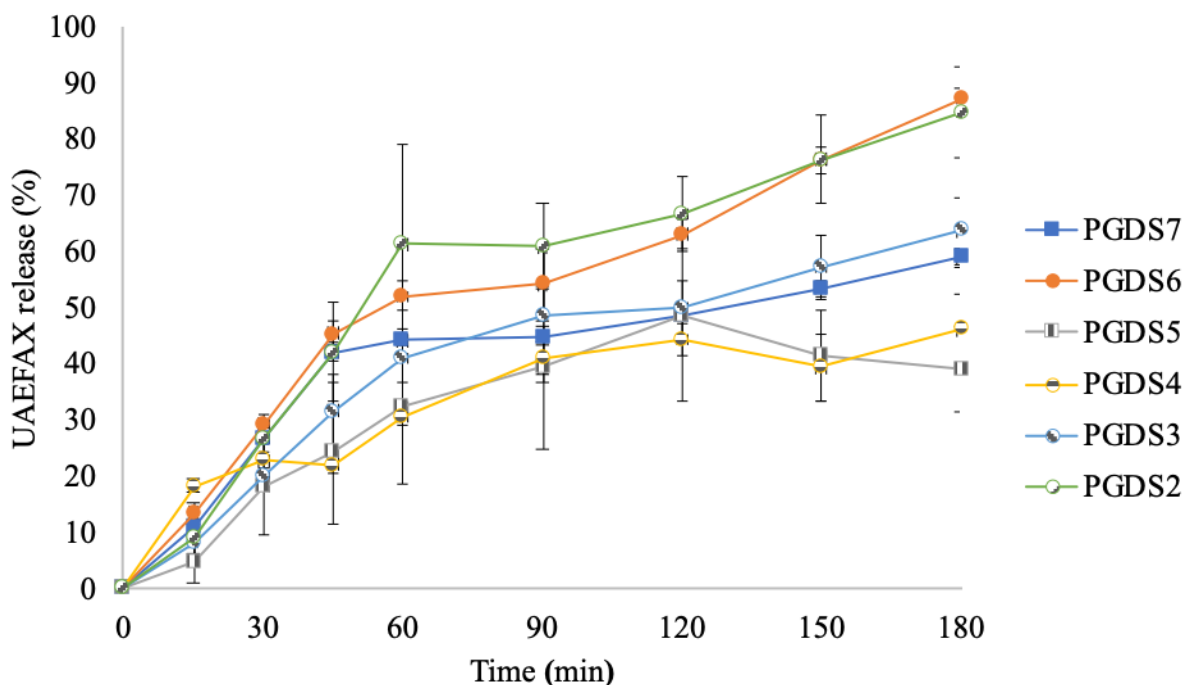


Figure 3.14. Release characteristics of pectin-gelatin based delivery systems loaded with FOCAXUAE in simulated intestinal fluid (SIF pH 6.8). PGDS7, 3% high-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 3.55; PGDS6, 3% low-methoxyl pectin 15% gelatin gummies coated with HPMC (5% wt.) pH 2.9; PGDS5, 4% high-methoxyl pectin 30% gelatin gummies pH 2.9; PGDS4, 4% low-methoxyl pectin 30% gelatin gummies pH 2.8; PGDS3, 2% high-methoxyl pectin 8% gelatin gummies pH 2.8; PGDS2, 2% low-methoxyl pectin 8% gelatin gummies pH 2.85.

3.3.3 Micrographs of pectin-gelatin delivery systems

FIB-SEM images of the PGDSs revealed some physical attributes to explain the burst seen in the release profiles and further explained the releases characteristics observed. For instance, the lack of homogeneity and ordered distribution of the FOCAXUAE within the PGDS2, PGDS3, PGDS4, and PGDS5 explains the trends seen in the release profiles. Perhaps, the lack of favorable interactions occurring within the colloid system during preparations resulted in the separating out of the bioactive compounds, coalesced lipid droplets (arrows in Figure 3.15A-D), however, with PGDS3 and PGDS5, Figure 3.15B and D, there are fewer formless globules seen. With the formulations of PGDS2-5 samples with consistent release characteristics among the treatments (n=5) could not be generated. However, with the addition

HPMC, better release characteristics were seen and apparent by the structures seen in the micrographs below, Figure 3.11. E and F (arrows show double encapsulated FOCAXUAE).

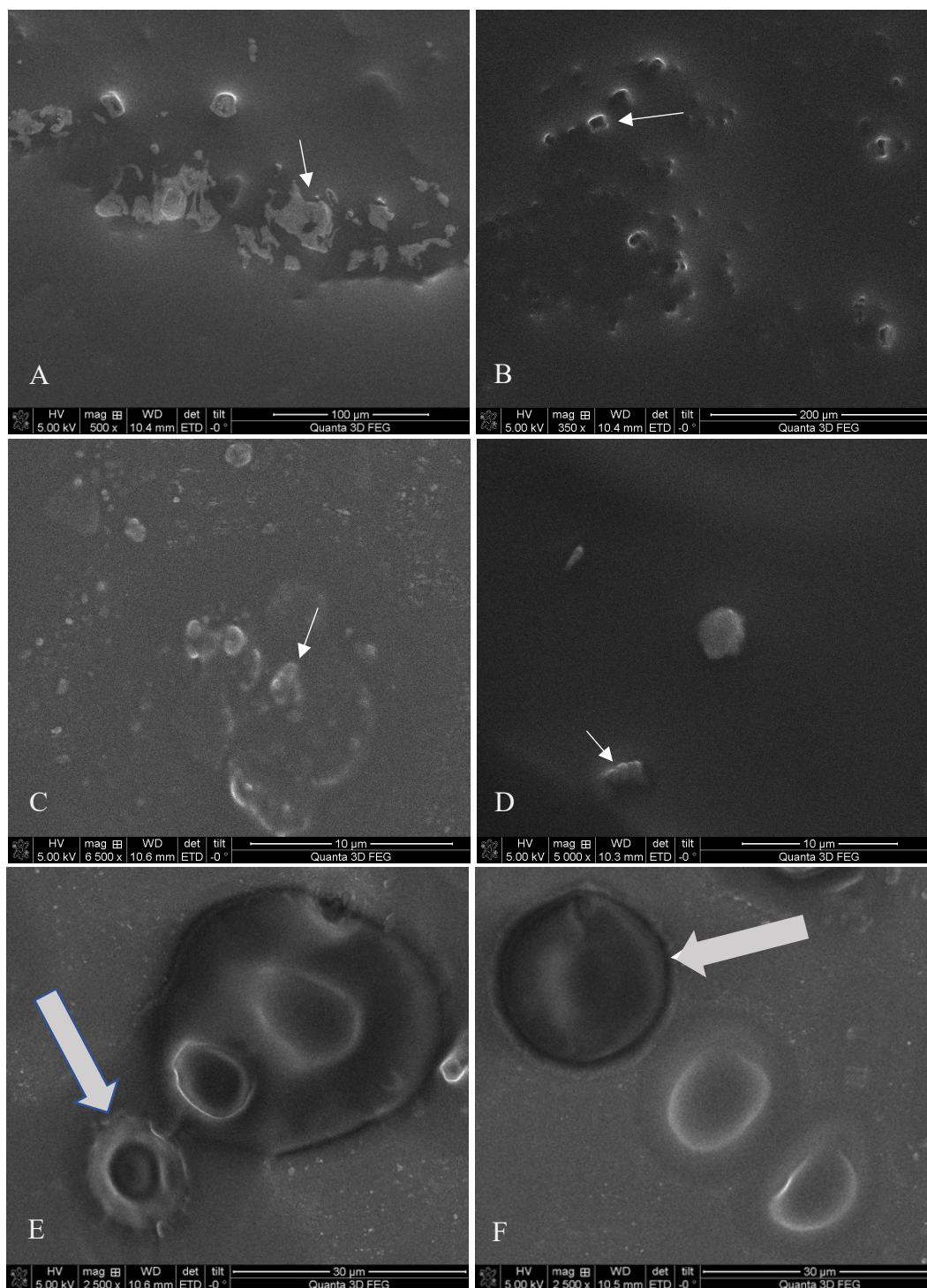


Figure 3.15. Scanning electron micrograph of cross-section of PGDS sample, after freeze drying (350 to 3,500x magnification). PGDS2 (A) - 2% LMP:8% G, pH 2.85 (5%); PGDS3 (B) - 2% HMP:8% G pH 2.8 (5%); PGDS4 (C) - 4% LMP:30% G pH 2.8 (8%); (caption cont'd).

PGDS5 (D) - 4% HMP:30% G pH 2.9 (8%); PGDS6 (E) - 3% LMP:15% G:5% HPMC pH 2.9 (10%); PGDS7 (F) - 3% HMP:15% G:5% HPMC pH 2.9 (10%). Shaded arrows in E and D indicate double encapsulation.

Initially, it was expected that LMP would be more compatible with gelatin and would form a single-phase system, compared to HMP and gelatin. However, the inability of LMP in encapsulating FOCAXUAE and the lack of gel formation proved otherwise. Furthermore, the gel forming capabilities of HMP were not affected by the presence of a second or third polymer. However, the presence of HPMC did induce the formation of capsules. Studies have reported the ability of high concentrations of Na⁺ and K⁺ ions induce gelation, regardless of alkali hydroxides NaOH and KOH preventing gelation due to demethoxylation (Wehr, Menzies, & Blamey, 2004). This explains the inability of LMP to retain a gel in SIF.

3.3.4 Characterization of the PGDS

The FOCAXUAE is encapsulated in a double emulsion of pectin and HPMC within the gelatin network that appeared heterogeneous in size, PGDS6 and PGDS7, Figure 3.15E and F, respectively. However, in the formulation with HMP, Figure 3.15F, there was greater uniformity among the size of the capsules. The PGDS without HPMC showed less uniformity, as there were FOCAXUAE globules (formless shapes) of FOCAXUAE in cross sections of the freeze-dried hydrogels (Figure 3.11A-C) or a small number of FOCAXUAE droplets (Figure 3.15D). The SEM images obtained are representative of high encapsulation efficiency, which was $94 \pm 1\%$ for the three samples.

Moreover, high-methoxyl pectin had a high encapsulation efficiency (89.2%) and contributed to the controlled release of FOCAXUAE in both SIF and SGF with and without HPMC. HMP solutions gel because of hydrophobic interactions and hydrogen bonding between pectin molecules, while LMP solutions require a sufficient concentration of divalent cations to gel. The initial burst release (within the first 15 minutes) is thought to be caused by poorly

entrapped FOCAXUAE, weak network formation from highly hydrated and charged carboxylate groups, or the FOCAXUAE adsorbed onto the outside of the particles. The very glossy surface of the PGDS cubes is because the amount of regular reflection from the surface is high and the amount of diffuse reflection is low. In contrast, HPMC capsules have a visually matt surface with a greater amount of diffuse reflection, suggestive of a more irregular surface. This may imply possible formation of an enteric barrier around the PGDS gummies

The PGDS (PGDS6 and PGDS7) prepared with HPMC (%5 wt./wt. of final volume) resulted in a delay of the burst release of FOCAXUAE, which can be attributed to a film coating developing around the delivery system when exposed to a near neutral solution. Release of AX from the PGDS matrix was investigated and, in an effort to examine the potential of the pectin-gelatin gummy confectionary as a lymphatic delivery system release characteristic were evaluated.

Despite the high concentration of sugar, it appears that HPMC particle swelling and coalescence may not have been suppressed, as it was in a study by Williams, Ward, Hardy, and Melia (2009). Based on data obtained from the swelling study, PGDS with HPMC (5% wt.) had extensive swelling for 180 min in SIF, but not in SGF. It is hypothesized that disintegration occurred after the three-hour exposure to SIF during the swelling study, as it did for the other formulations in both SGF and SIF. That hypothesis is validated by the absence of a burst release phenomena when evaluating the release profiles. The swelling studies in SGF showed that PGDS formulated without a concentration of HPMC to produce an enteric coating disintegrated rapidly. It should be noted that an enteric coating may possibly hinder the intestinal uptake and absorption of AX in the lymphatic system. Consequently, the gel barrier was not capable of tolerating the low pH of the SGF, thus making AX available. Aside from the subtle

swelling occurring, in the SGF, you can see that there is less burst of oil release from PGDS6 and PGDS7. In all other formulations, there was no gel layer formation on the surface of the PGDS, therefore the matrix had immediate release characteristics. Weaker gel layers were formed in PGDS2, PGDS3, PGDS4, and PGDS5, as evidenced by the fact that no data could be collected from the swelling study. In PGDS formulated without HPMC, the release of FOCAXUAE was unavoidable. Looking at the formulations combining gelatin with HMP, there was an associative interaction between the two polymers and neither component had an effect on the other. The gel strength of PGDS4, PGDS5, PGDS6 and PGDS7 was evidenced by their firmness when compressed between the index and thumb fingers and their retained shape (by visual inspection).

HMP gels when the molecules are hydrated, and subsequently lose some of the water (dehydration) due to the presence of co-solutes, i.e., high concentrations of sugar. Dehydration causes the pectin molecules to form hydrogen bonds between chains, resulting in a decrease in electrostatic forces (decrease in cross-linking by divalent cations). Those hydrogen bonds create a network to trap the remaining water and solute molecules. In this case, PGDS2 and PGDS5, despite the inability of the pectin to encapsulate the FOCAXUAE, the properties of HPMC within the PGDS6 and PGDS7 formulations create the conditions necessary to form a network to protect the bioactive compound. The resulting formulation contained a double emulsion.

3.4 Conclusion

A hydrocolloid delivery system, which incorporated the astaxanthin (in flaxseed oil) into edible gummies, was designed, developed and tested. The delivery system was tested in simulated gastric fluid (SGF) and simulated intestinal fluid (SIF), and performed well. It withstood SGF for typical stomach transit times and released the flaxseed oil (solvent) containing astaxanthin within normal GI tract times when tested in the simulated intestinal

environment. Rapid release of FOCAXUAE from pectin:gelatin:HPMC delivery systems was prevented by the presence of the cellulose derivative HPMC. In addition to not encapsulating FOCAXUAE, the other formulations were not capable of maintaining their gel structure in the SGF media, purporting that the necessary conditions to sustain such gelation were not met. The cation concentration in either the SGF or the SIF media were not in concentrations great enough to elicit an ionotropic gelation mechanism with the LMP. However, one must consider the that de-esterified LMP is unable to form gels without the presence of calcium. Therefore, no sufficient gel structures were formed at a low pH. With an increase in the pH (pH 6.8), which decreases the amount of a divalent cations (Ca^{+}) needed for gelation, gelling was observed in SIF, which contained calcium chloride. LMP is deesterified in alkaline environments, thus decreasing the requirement of a divalent cation for gelation (Garnier, Axelos, & Thibault, 1993). The results of this study showed that, potentially, the delivery system that was designed and developed using HMP or LMP, gelatin, and HPMC can be used for the controlled release of flaxseed oil containing astaxanthin.

CHAPTER 4 ASTAXANTHIN: CHEMOTHERAPEUTIC PROPERTIES

4.1 Introduction

The dietary intake of carotenoids derived from the marine environments provide numerous health beneficial effects such as antioxidant, antimicrobial, anti-inflammatory, and anti-cancer activities (Boominathan & Mahesh, 2015). Astaxanthin (AX), a ketocarotenoid, found throughout marine organisms, most notably particular interest to the crustacean species of Louisiana (shrimp, *Litopenaeus spp.* and red swamp crawfish, *Procambarus clarkii*). Beneficial properties of astaxanthin include; reducing the risk of heart disease, inflammation, and oxidative stress, in addition to a plethora of anti-cancer roles (Kowshik et al., 2014; Kurihara, Koda, Asami, Kiso, & Tanaka, 2002; Palozza et al., 2009; L. Zhang & Wang, 2015). The consumption of dietary carotenoids is reported to contribute to the inverse relationship between dietary intake and the risk of major clinical diseases, such as cancer.

Cancer has a global impact on people of all social classes. Breast cancer, the primary cause of cancer deaths in women, can be classified into estrogen receptors (ERs) +, human epithelial growth factor receptor 2 (HER2) +, and triple-negative breast cancer (TNBC) subtypes (Qu et al., 2014; T. Yoshida et al., 2014). Treatment of TNBC is the toughest obstacle for researchers because of its invasive and aggressive nature, as well there be fewer targeted medications. This metastatic characteristic of TNBC is the reason for it having the lowest survival rate of any other subtype of breast cancer. Very few drugs have been approved for the treatment of TNBC. Treatments for metastatic TNBC are centered around improving the quality of life through reduction of the cancer-related symptoms, as opposed to providing a cure, the primary goal of treatment for patients with localized breast cancers (Zeichner, Terawaki, & Gogineni, 2016).

Over the past years, carotenoids have been delivered to cells in culture incorporated in liposomes, water-miscible beadlets or mixed micelles (O'Sullivan, Woods, & O'Brien, 2004). Additionally, organic solvents have been used as delivery vehicles for carotenoids, despite classic difficulties of insolubility, instability, and cytotoxicity being related to solvent use.

In this study, the effect of ultrasound assisted extracted astaxanthin in flaxseed oil (FOCAXUAE) from crawfish processing byproducts (CB) delivered to cells in culture on cell density and the cell propensity of MDA-MB-231 cells, a triple-negative breast cancer cell line, to undergo an epithelial-mesenchymal transition (EMT), was investigated.

4.2 Materials & Methods

4.2.1 Emulsions with and without astaxanthin preparation for cell treatment

Astaxanthin was extracted by the method described above in section 2.2.3.2. of Chapter 2, with some modification. Ethanol (200 g) used for extraction of CB (25 g). Three hundred grams of ethanol was used to extract astaxanthin from a total of 100 g of CB; this includes washing and filtration procedures. Astaxanthin content in the ethanol extracts was determined based on the equation adopted from (Takeungwongtrakul & Benjakul, 2016). For determination of astaxanthin content, 5 g of FO was added to a 100 ml aliquot of the ethanol with astaxanthin extract.

Four amphiphilic surfactants, tween 20 (T20), tween 80 (T80), triton X (TX), and sunflower lecithin (SL), were used to emulsify the oil-in-water (flaxseed oil in DMEM) formulations to deliver astaxanthin extracted CB *in vitro*. Surfactant was mixed with DMEM to prepare a 1:100, 1:1000 or 0.5:1000 dilutions. The mixtures were sonicated for 60 s using an ultrasonic processor (Model CPX 500, Cole Palmer Instruments, Vernon Hills, IL, USA) with a 2mm stepped microtip probe threaded to the 10 mm probe of an ultrasonic booster horn fitted to an ultrasound converter for producing sonic waves at a frequency of 20 kHz electrical energy.

Following surfactant selection, emulsions were prepared by adding oil (100mg, 10%w/v) to 1mL of DMEM. Then 25 mg of emulsifier were added (2.5% w/v). Then, the solution was vortexed at 3000 rpm for 1 min before further emulsification using an ultrasonic processor (Model CPX 500, Cole Palmer Instruments, Vernon Hills, IL, USA) with a 2mm stepped microtip probe. This microtip probe was threaded to a 10mm horn fitted on an ultrasonic converter, for producing sonic waves at a frequency of 20 kHz. Emulsions were prepared in a 5 ml amber Eppendorf to prevent photooxidation.

4.2.2 Emulsion Stability

Emulsions prepared by adding oil (100mg, 10%w/v) to 1mL of DMEM, then adding 25 mg of emulsifier (2.5% w/v). This solution was vortexed at 3000 rpm for 1 min before further emulsification using an ultrasonic processor (Model CPX 500, Cole Palmer Instruments, Vernon Hills, IL, USA) with 13 mm probe connected to an ultrasound converter for producing sonic waves at 20% power of 20 kHz electrical energy. Similar to the parameters used for preparing emulsions for treatments. To measure turbidity at 500 nm 10ul of emulsion was added to 3 mL of 0.1% sodium dodecyl sulfate (SDS, a 300-dilution factor) in cuvettes immediately after emulsion preparation. Other data not shown.

Following surfactant selection, emulsions were prepared by adding oil (100mg, 10%w/v) to 1mL of DMEM. Then 25 mg of emulsifier were added (2.5% w/v). Then, the solution was vortexed at 3000 rpm for 1 min before further emulsification using an ultrasonic processor (Model CPX 500, Cole Palmer Instruments, Vernon Hills, IL, USA) with a 2mm stepped microtip probe. This microtip probe was threaded to a 10mm horn fitted on an ultrasonic converter, for producing sonic waves at a frequency of 20 kHz. Emulsion were prepared in a 5 ml amber Eppendorf to prevent photooxidation.

4.2.2 Cell culture

The human breast cancer cell, MDA-MB-231 (passages 30–32) was cultivated in 100-mm-diameter tissue culture flask. Cells were maintained in 5% CO₂ at 37°C and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco BRL, Grant Island, NY) supplemented with 10% Hyclone Cosmic Calf Serum (HyClone Laboratories, Utah), 50 ng/mL insulin (Sigma-Aldrich, St. Louis, MO, USA), and 1% MEMAA, NEAA, sodium pyruvate and antibiotic-antimycotic (Gibco, Dublin, Ireland). For experiments, MDA-MB-231 cells were seeded at a low density (5×10^3 cells/well) into a 96-well plate.

4.2.3 MDA-MB-231 cell density

To examine the anticancer effects of astaxanthin emulsions (AXE, 3μM, 5μM, and 7μM) on MDA-MB-231 cells, a 96-well plate was incubated for 2 days, before being treated. Following incubation period, AXE was carefully removed from wells containing cells. Cells were stained with 33% crystal violet, 20 μL per well, for 15 minutes on a rocker then washed and allowed to dry (2 hours). After drying, cells were either assessed for morphological characteristics or stains eluted for relative cell viability (RCV) per section 4.2.3. The cells were imaged using an inverted fluorescence microscope (Nikon Eclipse Ti2), and NIS Elements Advanced Research Microscope Imaging Software (NIS Elements AR, Nikon).

4.2.4 *In vitro* assessment of astaxanthin exposure from AX emulsions on MDA-MB-231 cells for cell viability

The viability of the MDA-MB-231 and cellular contact with AXE was evaluated using absorbance measurements. The absorbance indicated relative cell viability (RCV) in regards to the relative number of total cells (fixed cells with uncompromised membranes to retain the crystal violet dye) in a one well compared to another. The emulsions diluted with DMEM was carefully removed from cell monolayers, then stained with crystal violet was added to the wells

containing cell monolayers and placed on a rocker for 15 minutes. Subsequently, the plates were rinsed of the stain with water and allowed to dry (2 hrs) RCV was evaluated numerically by measuring the optical density (absorbance at 570 nm) with the cell imaging multi-mode plate reader Cytation™ 5 (Biotek, Winooski, VT). RCV was calculated based on the equation 4.1:

$$\text{Relative cell viability} = \frac{(\text{average of treated wells absorbance-blank})}{(\text{average of control wells absorbance-blank})} \times 100 \quad (\text{Eq. 4.1})$$

4.2.5 Statistical Analysis

All data were analyzed with GraphPad Prism 4.0 software (GraphPad Software, Inc.; San Diego, CA, USA). The RCV was evaluated by one sample t-test and Wilcoxon test at a significance level of 0.05 and plotted by GraphPad Prism.

4.3 Results & Discussion

The genomic characteristics and nature of TNBC cells make them difficult to target and treat. The utilization of a natural anti-cancer agent, like astaxanthin, is important for improving upon the adjuvant treatments available for TNBC patients. This not only reduces the side effects of systemic therapies routinely used, it also improves upon the quality of life during recovery. Over the past years, carotenoids have been delivered to cells in culture incorporated in liposomes, water-miscible beadlets or mixed micelles (O'Sullivan et al., 2004). Additionally, organic solvents have been used as delivery vehicles for carotenoids, despite classic difficulties of insolubility, instability, and cytotoxicity being related to solvent use.

During preliminary tests, a visual confluency estimate was used to assess the effects of the four surfactants to emulsify DMEM and flaxseed oil on the viability MDA-MB-231 cells. Multiple variations in the concentration of surfactants (i.e., high, medium, and low) SL, T20, T80, and TX were assessed. Sunflower lecithin (SL) was found to show minimal deleterious effects on the viability of MDA-MB-231 cells, as seen in Figure 4.1. Various concentrations

were tested to determine the ideal amount of surfactant to achieve the optimal response from the MDA-MB-231 cells as it relates to viability. Visually inspections show that T20 and TX have a greater anti-proliferative or apoptotic effect in terms of cell density than do T80 and LC on MDA-MB-231 cells. Some results not pictured.

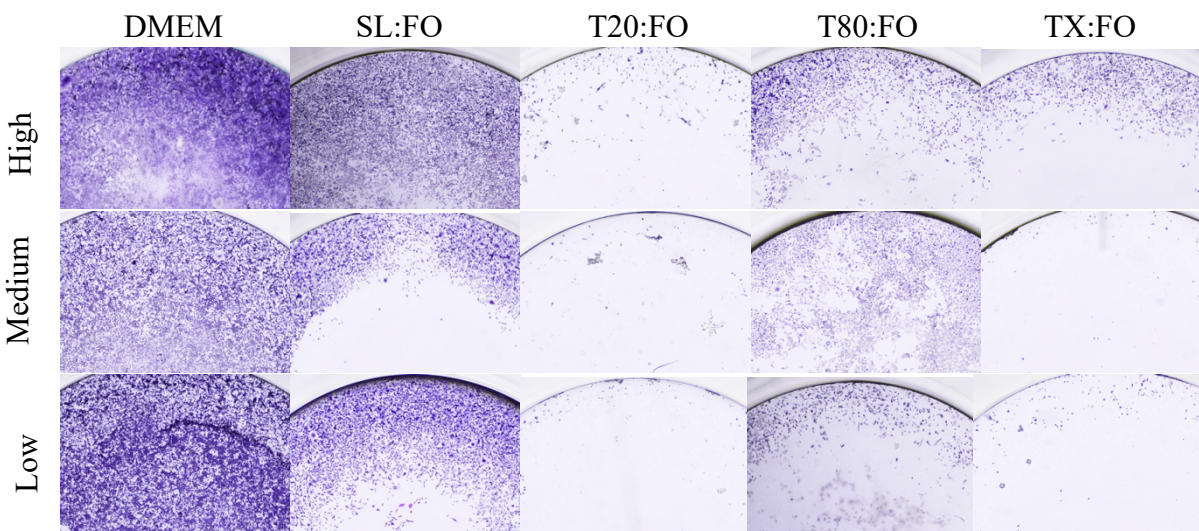


Figure 4.1. Visual confluency estimation to surfactant and optimization. Microscopy using a Nikon Eclipse Ti2 inverted fluorescence microscope at 2x magnification.

Once the dosages optimize with a suitable concentration of surfactant to use in delivering AX *in vitro*, a closer assessment of the effects of surfactant/emulsifier on the relative cell number (% RCV) of MDA-MB-231 cells was assessed before proceeding with treatments. Sunflower lecithin showed a more representative RCV (79.37 ± 0.87) than the other surfactants, (T20 (8.6 ± 2.51), T80 (25.04 ± 3.00), and TX (17.11 ± 3.00)), shown in Figure 4.2. Also, considering the ultimate goal of the study is to utilize natural rather than synthetic compounds to attenuate the deleterious effects of TNBC and the adjuvant therapies available.

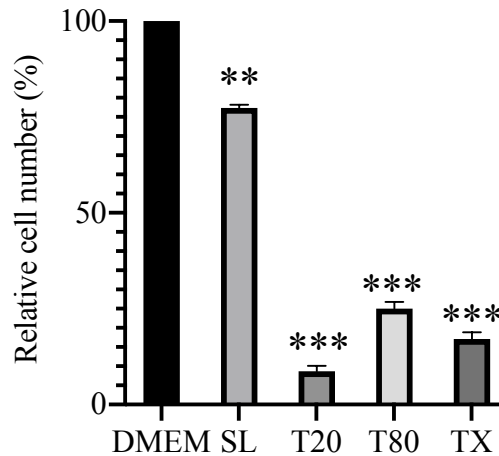


Figure 4.2. MDA-MB-231 % cell viability in different surfactants. Means \pm SEM of triplicate determinations. ** $p < 0.01$, *** $p < 0.001$. DMEM = Dulbecco's modified Eagle's medium. SL = lecithin. T20 = Tween 20. T80 = Tween 80. TX = Triton X.

AX emulsions (AXE) were prepared in DMEM with 10% sunflower lecithin (SL) at concentrations of 0.0025:0.005% to 0.1:0.2% (LD:AXE, wt:wt). Controls prepared at same concentrations as LD:AXE, using flaxseed oil without AX. MDA-MB-231 cell line was seeded at 5×10^3 cells/well in 96-well plates and incubated at 37 °C for 24 hours. Cells were then treated with 100 μ L/well LD:E or LD:AXE. LD:AXE treated cells (image b, 1-9) displayed a mesenchymal-to-epithelial transition (MET) compared to control treated cells (image a, C and E), which suggests a less metastatic and aggressive phenotype. LD:AXE treatments resulted in a decrease in cell number in a somewhat dose-dependent (3 μ M, 5 μ M, and 7 μ M AX) manner, seen by RCV decreasing by 46.31%, 42.2%, and 55.51%, respectively, from the normalized value (Figure 4.3).

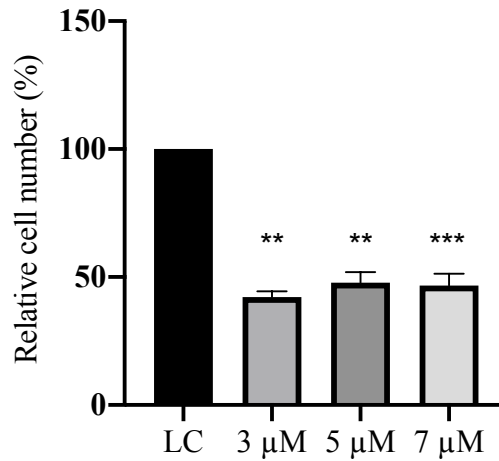


Figure 4.3. MDA-MB-231 viability of treated cells. Means \pm SEM of triplicate determinations. ** $p < 0.01$, *** $p < 0.001$. LC = lecithin control. 3 μ M = LC with 3 μ M astaxanthin. 5 μ M = LC with 5 μ M astaxanthin. 7 μ M = LC with 7 μ M astaxanthin.

The morphology of astaxanthin treated cells compared to untreated cells showed a decreased number of cells resembling a mesenchymal-like phenotype (Figure 4.4. b) 1-9). This is suggestive of a mesenchymal-to-epithelial transition (MET), or reversal of EMT. EMT has been reported to be the cause of the invasive, metastatic, and multi-drug resistant nature of cancer cells (Chaffer, San Juan, Lim, & Weinberg, 2016; Du & Shim, 2016; Little et al., 2016; Milano, Muthuswamy, & Asthagiri, 2016; Pastushenko & Blanpain, 2019). In this study, LC:FO alone did not significantly affect MDA-MB-231 cell viability or MET. Thus, indicating the possibility that neither LC or FO possess any cytotoxic or genotoxic activity in the levels used.

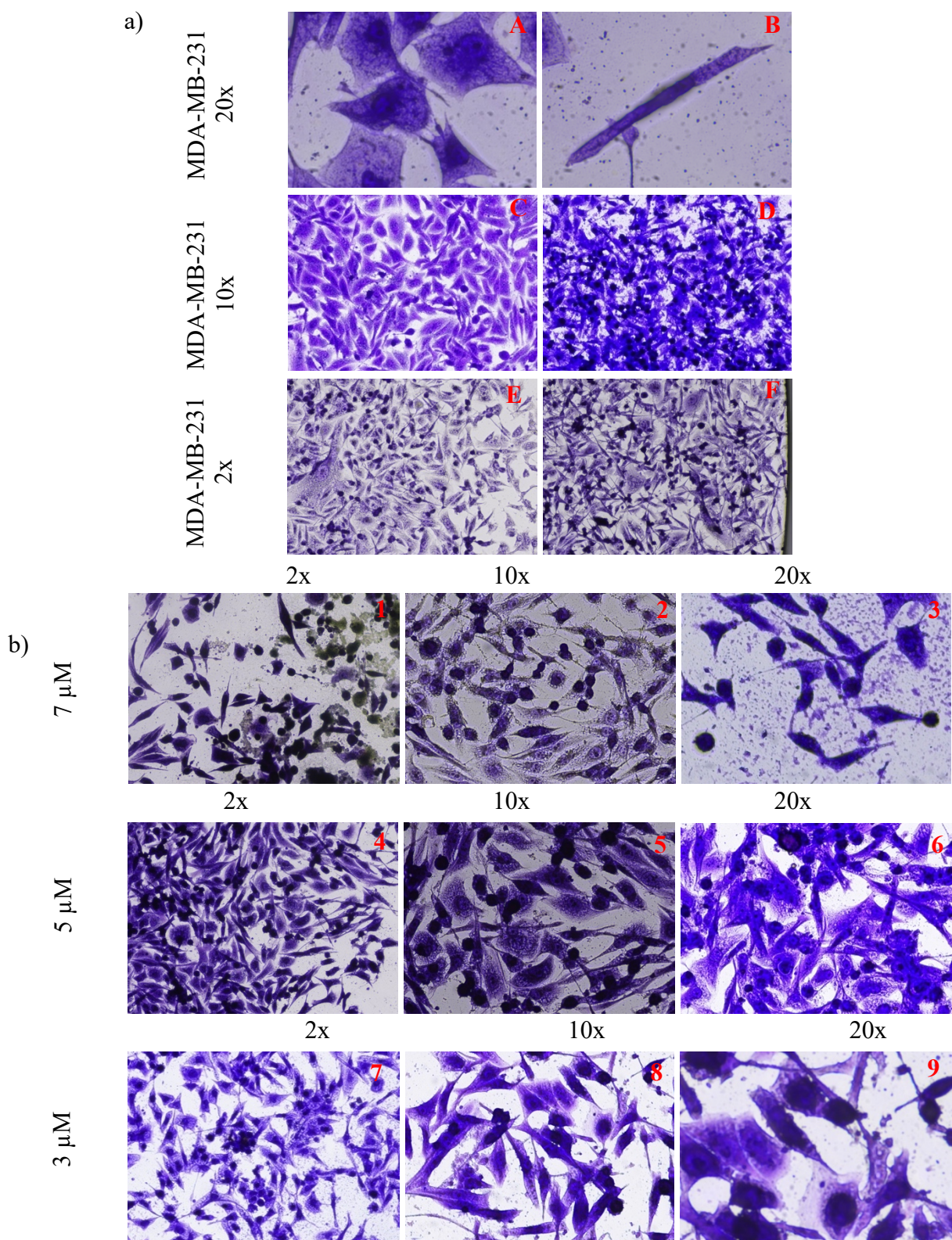


Figure 4.4. Evaluation of astaxanthin on the morphology of MDA-MB-231 triple-negative breast cancer cell line *in vitro* by darkfield microscopy. Microscopy using a Nikon Eclipse Ti2 inverted fluorescence microscope.

Microscopy revealed (Figure 4.4) MDA-MB-231 cells displayed (A) epithelial and (B) mesenchymal-like, spindle-shaped cells. (C, E) Control MDA-MB-231 cells in DMEM and (D, F) treated MDA-MB-231 cells by brightfield microscopy. MDA-MB-231 cells grew as sheet-like monolayers (indicated by arrow) of tightly packed colonies. (Image b) MDA-MB-231 cells in flaxseed oil DMEM emulsion with astaxanthin (AX) increasing in concentration appears to increase cell to cell interactions (4-9), compared to the loss of cell to cell interaction at 7 μ M treatments (1-3). MDA-MB-231 cells treated with AX at 3 μ M, 5 μ M, and 7 μ M concentrations exhibit a reduction in cells displaying mesenchymal-like morphology. MDA-MB-231 cells treated with 5 μ M astaxanthin had an increase in cell population (4), compared to those treated with 7 μ M (1-3) having small rounded cells with a globular appearance (possibly dead with debris). Numerous MDA-MB-231 cells are egg-shaped and flattened with characteristic morphologies resembling epithelial cells. Some cells show filopodia (8). Different concentrations of AX exert varying degrees of cell to cell adhesion (4,7) and dissemination (1). More importantly, spindle-like cells with mesenchymal morphology appear to be reduced in a dose dependent manner.

In addition to cells gaining motility and invasiveness when they undergo an EMT, they also develop novel interactions with the microenvironment. Such interactions lead to the migration of cancer cells from one site to the next.

4.4 Conclusion

The studies reported here demonstrate that AX exerts anticancer effects on MDA-MB-231 cells *in vitro* with respect to cell viability and morphology. The RCV of MDA-MB-231 was reduced. It can be seen that the incorporation of SL as a vehicle for administration of the bioactive compound AX, has a significant effect on the growth or phenotypic changes to MDA-

MB-231 cells based on the RCV (Figure 4.3). The same can be said about flaxseed oil. Additionally, MET was suggested to be induced *in vitro*, thereby decreasing the TNBC metastasis and possibly prolonging survival. However, an *in vivo* analysis would have to be performed before such correlation can be accepted. These results provide insight on the potential application of crawfish derived astaxanthin, as a potential therapeutic agent in TNBC treatment.

CHAPTER 5 CONCLUSION

Astaxanthin (AX) was extracted from crustacean processing byproducts, shrimp processing byproducts (SB) and crawfish processing byproducts (CB), using flaxseed oil. Higher extraction yields of AX is achieved using CB. Extraction of AX from CB in a continuous ultrasound assisted extraction (UAE) system yielded even greater recoveries than UAE of AX from SB and the conventional extraction method from SB and CB. Effects of conventional extraction and UAE of AX from SB and CB on the oxidation and stability of the flaxseed oil were evaluated. The UAE of AX extracted from CB had a greater antioxidant capacity, than other extracts tested. UAE was effective for increasing the recovery of AX from CB and preventing a loss in the quality of the flaxseed oil used as an extraction solvent. The UAE method was also effective for concentrating AX to be used in the design and development of a delivery system. The delivery system, composed of three polymers, hydroxypropyl methylcellulose (HPMC), gelatin (G), and pectin (P), was used to encapsulate flaxseed oil containing AX (FOAX). The polymeric delivery system was capable of delaying the release of the FOAX extracted CB in simulated gastrointestinal tract fluids by several mechanisms afforded by the polymers (HPMC as an enteric coating, G gelling properties to slow release, and P gelling properties in ionic solutions to slow release). Additionally, AX extracted from CB showed anticancer properties against a triple negative breast cancer (TNBC), MDA-MB-231 cells *in vitro*. Exposure of the MDA-MB-231 cells to AX altered cell morphology. A greater number of cells exposed to AX reversed their epithelial-to-mesenchymal transition (EMT), representing a less aggressive phenotype of TNBC MDA-MB-231. Nonetheless, an *in vivo* analysis needs to be performed prior to a correlation between a mesenchymal-to-epithelial transition (MET) and decreased metastasis with increased life expectancy in human subjects.

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